

**Fate of fluorinated organic pollutants in aquatic plant systems: Studies
with Lemnaceae and Lemnaceae tissue cultures**

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The Academic Faculty

by

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**Fate of fluorinated organic pollutants in aquatic plant systems: Studies
with Lemnaceae and Lemnaceae tissue cultures**

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To Michael

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	IV
LIST OF TABLES	XI
LIST OF FIGURES	XII
LIST OF SYMBOLS	XVI
LIST OF ABBREVIATIONS	XVIII
SUMMARY	XXIII
CHAPTER 1: INTRODUCTION	1
CHAPTER 2: LITERATURE REVIEW	4
Wetlands for treatment and reclamation of wastewaters	4
The duckweed family, Lemnaceae	9
Parameters affecting Lemnaceae growth and reproduction	11
Phytotoxicity assessments using Lemnaceae species	14
Fate of Organic Pollutants in Aquatic Plant Systems	17
Plant uptake of organic pollutants	17
Phytometabolism of organic pollutants	21
Fate of plant metabolites of organic pollutants	33
Plant tissue culture	35
History of plant tissue culture	35
Basics of plant tissue culture	36
Plant tissue culture of Lemnaceae	42
Plant tissue cultures to study pollutant metabolism and phytotoxicity	45
Descriptions of organic pollutants	48
Fluorinated phenols	56

Fluorophenols	57
Trifluoromethylphenols (TFMeP)	60
Named organic pollutants	61
2,4-Dichlorophenoxyacetic acid (2,4-D)	62
3-Trifluoromethyl-4-nitrophenol (TFM)	63
Ibuprofen	64
Fluoxetine	65
Clofibric acid	68
DEET	69
Triclosan	70
CHAPTER 3: UPTAKE OF FLUORINATED PHENOLS BY LEMNACEAE	74
Introduction	74
Material and Methods	76
Collection and Maintenance of Lemnaceae	76
Uptake of Fluorinated Phenols by <i>L. minor</i>	77
Statistical Analyses	79
Results and Discussion	80
Uptake of Fluorinated Phenols by <i>L. minor</i>	80
Effects of Fluorinated Substituents	81
Effects of Partitioning-Related Properties	86
Effect of nitro- substituents on fluorinated phenol uptake	88
Summary	90
CHAPTER 4: CONCENTRATION EFFECTS ON UPTAKE AND PLANT ACTIVITY IN <i>LEMNA MINOR</i> SYSTEMS	91
Introduction	91
Materials and Methods	93

Materials and supplies	93
Oxygen production rate (OP) assessment	94
Uptake and toxicity assessments	95
Results and Discussion	97
Oxygen production rate (OP) assessment	97
Plant density effects on oxygen production rate (OP)	99
Plant activity of <i>L. minor</i> exposed to 3-TFMeP and 3-FP	103
Removal of 3-TFMeP and 3-FP	106
Summary	109
CHAPTER 5: TEMPERATURE EFFECTS ON UPTAKE, PLANT ACTIVITY, AND SORPTION IN <i>LEMNA MINOR</i> SYSTEMS	111
Introduction	111
Materials and methods	112
Results and Discussion	113
Uptake of fluorinated organic pollutants	113
Temperature effects on sorption	115
Temperature effects on uptake	117
Temperature effects on inhibition and plant activity	119
Summary	121
CHAPTER 6: UPTAKE OF WASTEWATER-ASSOCIATED ORGANIC POLLUTANTS BY LEMNACEAE	122
Introduction	122
Materials and methods	123
Collection and maintenance of Lemnaceae	123
Chemicals and stock preparations	124
Experimental reactors and controls	126

Results and discussion	129
Ibuprofen	131
Fluoxetine	136
Clofibric acid	139
Meta-N,N-diethyl toluamide (DEET)	139
Triclosan	141
2,4-Dichlorophenoxyacetic acid (2,4-D)	144
Effects of hydrophobicity on uptake and sorption	146
Summary	147
CHAPTER 7: INDUCTION OF LEMNACEAE CALLUS CULTURES	150
Introduction	150
Materials and Methods	152
Basal nutrient media and phytohormones	152
Collection of Lemnaceae species	152
Disinfection of Lemnaceae species	153
Callus induction methods	154
Assessment of callus induction frequency	158
Initiation of cell suspension from callus cultures	159
Results and Discussion	160
Disinfection of Lemnaceae species and subsequent growth of frond lines	160
Callus induction in <i>L. minor</i> and <i>L. punctata</i>	162
Initiation of cell suspension cultures	170
Summary	170
CHAPTER 8: TOXICITY AND METABOLISM IN LEMNACEAE AND LEMNACEAE CALLUS CULTURES	173
Introduction	173

Material and Methods	176
Collection and callus initiation of Lemnaceae	176
Fluorinated organic pollutants	177
Oxygen production rate (OP) assessment	178
Callus toxicity assessment	180
Identification of plant metabolites of 3-TFMeP	183
Results and Discussion	184
Characterization of control callus growth	184
Intercomparisons of callus toxicity responses	187
Comparisons of callus culture and OP responses	194
Effects of pH on toxicity of fluoxetine to Lemnaceae plants	198
Phytometabolism of 3-TFMePh in Lemnaceae plants and callus	199
Summary	203
CHAPTER 9: CONCLUSIONS AND RECOMMENDATIONS	204
Recommendations for future research	210
REFERENCES	212

LIST OF TABLES

Table 2.1. Removal of wastewater constituents by 21 constructed treatment wetlands in North America. Data from (Kadlec 2003).	5
Table 2.2. Examples of toxicity assessments using Lemnaceae species.	15
Table 2.3. Examples of reports of phytometabolism of organic pollutants by terrestrial and aquatic plants.	27
Table 2.4. Common hormones and phyto regulators used in plant tissue cultures	39
Table 2.5. Experimental organic pollutants: Structures and relevant chemical properties.	51
Table 2.6. Select trifluoromethyl-substituted aromatics and their uses.	57
Table 5.1. Fitted Arrhenius parameters for uptake of fluorophenols (FP) and trifluoromethylphenols (TFMeP) by <i>L. minor</i> .	118
Table 6.1. Physicochemical properties of experimental organic pollutants and solvents used for stock preparations.	125
Table 6.2. Summary of results from active Lemnaceae reactors and controls.	148
Table 7.1. Phyto regulators used for callus induction and maintenance studies with chemical suppliers and solvents used for stock solutions.	152
Table 7.2. Summary of callus induction frequency and brief observations of callus growth for experimental methods.	163
Table 8.1. Representative parameters for description of toxicity responses of <i>L. minor</i> and <i>L. punctata</i> to fluorinated organic pollutants, as determined by callus and oxygen production (OP) rate assessments.	188

LIST OF FIGURES

Figure 2.1. Mechanisms for organic pollutant removal in wetland systems.	5
Figure 2.2. Cladogram of Lemnaceae species based on morphological, flavonoid, allozyme, and DNA sequence data.	10
Figure 2.3. Depiction of plant metabolism of organic pollutants (“xenobiotic”).	22
Figure 2.4. Depiction of uptake of 2,4-dichlorophenol by <i>L. minor</i> .	24
Figure 2.5. Schematic of degradation pathways for fluorinated phenols in <i>Rhodococcus</i> species from Boersma et al. (2001).	59
Figure 2.6. Biological (solid arrows) and abiotic (dashed arrows) degradation of 2-trifluoromethylphenol in incubations containing <i>Bacillus thermoleovorans</i> A2 and chloride-free Luria Bertani medium.	61
Figure 2.7. Proposed pathway for metabolic degradation of ibuprofen by <i>Sphingomonas</i> sp. strain Ibu-2.	65
Figure 2.8. Proposed photodegradation pathway of fluoxetine leading to O-dealkylated products (I and II) and hypothesized carboxylic acid photoproduct (III).	67
Figure 2.9. Proposed metabolic degradation of DEET by <i>Pseudomonas putida</i> DTB.	70
Figure 2.10. Proposed photodegradation pathway of triclosan in waters.	72
Figure 3.1. Normalized aqueous concentration of 3-fluorophenol (3-FP), 3-trifluoromethylphenol (3-TFMeP), 4-trifluoromethylphenol (4-TFMeP), and 4-fluorophenol (4-FP) with time in batch reactors with 0.5 g <i>L. minor</i> .	81
Figure 3.2. Uptake rate coefficients for fluorinated phenols based on position of fluoro (F) and trifluoromethyl (TFMe) substituents determined through two experiments (Trial 1 and Trial 2).	82
Figure 3.3. Uptake rate coefficients for fluorinated phenols based on substituent position .	84
Figure 3.4. Uptake rate coefficients versus fraction of halogenated phenol protonated at a pH representative of intercellular pH (pH 7.0).	87
Figure 3.5. Aqueous concentration of 3-trifluoromethylphenol (3-TFMeP), total nitrotoluenes (Total NT), and 3-trifluoromethyl-4-nitrophenol (TFM) with time.	89

Figure 4.1. Oxygen production rates for <i>L. minor</i> communities collected from Clayton County Water Authority treatment wetlands during June 2004 to July 2005.	95
Figure 4.2. Oxygen produced with time for carbonate concentrations of 1.8 mM, 5.8 mM, 6.8 mM, and 11.8 mM.	98
Figure 4.3. Effect of controlling pH variations with increasing phosphate buffer concentrations on O ₂ -produced by 0.5 g fresh mass <i>L. minor</i> .	98
Figure 4.4. Constant oxygen production (μmol/h) for 0.5 g <i>L. minor</i> for 2-14 h in three separate trials over 15 days.	100
Figure 4.5. OP by <i>L. minor</i> for increased initial plant mass in 19.6-cm ² surface-area reactor.	101
Figure 4.4. Response of normalized OP to increasing concentrations of 3-TFMeP (a) and 3-FP (b). OP is normalized to control (i.e., non-exposed) <i>L. minor</i> .	105
Figure 4.5. Uptake of 3-FP by <i>L. minor</i> at 22°C under continuous light.	107
Figure 4.6. a.) Change in pseudo-first-order uptake rate constants with concentration of 3-FP or 3-TFMeP. b.) Theoretical accumulation (μmol/g) of 3-FP or 3-TFMeP by <i>L. minor</i> in 24-h as calculated by Equation 3.	108
Figure 5.1. Percent oxygen in headspace of sealed reactors containing 0.5 g <i>L. minor</i> and 60 mL medium.	113
Figure 5.2. Normalized concentration of 2,3-difluorophenol with time in light and dark reactors.	114
Figure 5.3. Mass of 3-fluorophenol (3-FP) and 3-trifluoromethylphenol (3-TFMeP) sorbed to <i>L. minor</i> versus initial concentration at 22°C and 27°C.	115
Figure 5.4. Mass of 3-TFMeP sorbed to <i>L. minor</i> at temperatures ranging from 12°C to 35°C.	117
Figure 5.5. Arrhenius relationships for dependence of uptake rate coefficients on temperature for trifluoromethylphenols (TFMeP).	118
Figure 5.6. Normalized oxygen production rate (μmol/h-g) of 0.5 g <i>L. minor</i> exposed to 3-fluorophenol (3-FP) and 3-trifluoromethylphenol (3-TFMeP) for 24 h with temperature.	120
Figure 5.7. Oxygen production rate (OP) in μmol O ₂ /h-g <i>L. minor</i> of 0.5 g non-exposed <i>L. minor</i> with temperature.	120
Figure 6.1. Chemical structures of experimental organic pollutants.	124

Figure 6.2. Aqueous concentration of ibuprofen with time in experimental reactors and controls.	132
Figure 6.3. Chemical structures and molecular weights (M.W.) of ibuprofen and two isomers of hydroxy-ibuprofen.	132
Figure 6.4. Concentration of ibuprofen (left axis) and relative concentration of hydroxy-ibuprofen (right axis) with time in active Lemnaceae reactors and microbial degradation controls.	134
Figure 6.5. Linear aqueous depletion rates for ibuprofen in active reactors for 24 to 120 h versus Lemnaceae oxygen production rate at conclusion of exposure.	135
Figure 6.6. Aqueous concentration of fluoxetine with time in experimental reactors with initial concentrations of (a.) 10 μ M (<i>L. punctata</i>) and (b.) 5 μ M (<i>L. minor</i>).	137
Figure 6.7. Concentration of fluoxetine (μ M) with time (d) for active Lemnaceae reactors and control reactors with Lemnaceae inactivated by exposure to darkness (active dark control) or sodium azide (sorption dark control).	138
Figure 6.8. Aqueous concentration of clofibric acid (CFA) in reactors over 5 d (Trial 1: <i>L. minor</i>) and 16 d (Trial 2: <i>L. punctata</i>). Points represent means and error bars, not always visible, represent standard error of triplicate reactors.	140
Figure 6.9. Concentration of DEET (μ M) with time (d) in active Lemnaceae reactors and microbial degradation and physicochemical loss controls.	140
Figure 6.10. Aqueous concentration of triclosan with time in experimental reactors with initial concentration of 15 μ M.	141
Figure 6.11. Concentration of 2,4-DCP in active Lemnaceae reactors and controls exposed to triclosan.	142
Figure 6.12. Concentrations (μ M) of 2,4-dichlorophenoxyacetic acid (2,4-D) in experimental reactors and controls with time (d).	145
Figure 7.1. Outline of callus induction methods evaluated for <i>L. minor</i> and <i>L. punctata</i> from CCWA wetlands.	155
Figure 7.2. Growth and contamination of fronds after disinfection treatments.	161
Figure 7.3. Growth of axenic fronds on MS medium.	162
Figure 7.4. <i>L. minor</i> callus induced and maintained using the C method adopted from Chang and Chiu (1976).	165
Figure 7.5. <i>L. minor</i> callus induced with the S method based on Stefaniak et al. (2002).	166

Figure 7.6. <i>L. punctata</i> callus induced utilizing method developed by Li, et al. (2004).	169
Figure 8.1. Examples of <i>L. minor</i> , <i>L. punctata</i> 804/8, and <i>L. punctata</i> 815 callus (left to right, respectively).	178
Figure 8.2. Distributions of initial and final surface areas for <i>L. minor</i> , <i>L. punctata</i> 804/808, and <i>L. punctata</i> 815 in experimental controls (no exposure to fluorinated organic pollutant).	185
Figure 8.4. Comparison of normalized responses of <i>L. minor</i> callus to normalized responses of <i>L. punctata</i> 815, as represented by γ , for fluorinated organic pollutants.	193
Figure 8.5. Comparison of normalized responses of fronds (β) to normalized responses of callus cultures (γ) to fluorinated organic pollutants for <i>L. punctata</i> (a) and <i>L. minor</i> (b).	196
Figure 8.6. Normalized responses of <i>L. punctata</i> oxygen production rate (β) as affected by concentration of fluoxetine (μM) at pH 5.8 and pH 7.0.	199
Figure 8.7. Example chromatographic traces of (a.) <i>L. punctata</i> plants, (b.) <i>L. minor</i> plants, (c.) <i>L. punctata</i> callus cultures, and (d.) <i>L. minor</i> callus cultures exposed to 3-TFMeP.	201
Figure 8.8. Mass spectra of 3-TFMeP metabolite peak (retention time = 22.6 min) in <i>L. punctata</i> plant extraction.	202

LIST OF SYMBOLS

α	Plant activity, as measured by oxygen production rate (mmol O ₂ /h)
β	Normalized plant activity, demonstrating inhibition from organic pollutant exposure ($\beta = 1$ indicates no inhibition)
γ	Normalized callus growth factor
Θ	Arrhenius dimensionless temperature constant
σ	Hammett constant; used to describe electron withdrawing or donating nature of substituents
a	Generic fitting parameter
A_d	Final surface area of senescent callus
$A_{F, x \mu M}$	Final callus surface area at x μM
b	Generic fitting parameter
B	Biomass of <i>Lemna minor</i> in g dry mass/m ²
c	Empirical scaling constant for temperature-dependent growth of Lemnaceae
C_0	Initial aqueous concentration of fluorinated organic pollutant in experimental reactors and controls
C_I	Aqueous concentration of fluorinated organic pollutant 10 min after addition of Lemnaceae to experimental reactors and controls
$C_{i,50}$	Intercellular concentration at which 50% inhibition of <i>Lemna minor</i> activity was observed
C_{aq}	Aqueous concentration of organic pollutant in Lemnaceae
C_i	Intercellular concentration of organic pollutant in <i>Lemna minor</i>
$EC50$	Concentration at which 50% inhibition of endpoint is observed for given organism
h_B	Monod-like half-saturation value for biomass and <i>Lemna minor</i> growth (g dry mass/m ²)
$h_{M,OP}$	Monod-like half-saturation value for surface coverage and Lemnaceae oxygen production rate

h_N	Monod-like half-saturation value for nitrogen concentrations and <i>Lemna minor</i> growth (mg N/L)
h_P	Monod-like half-saturation value for phosphorus concentrations and <i>Lemna minor</i> growth (mg P/L)
k^*	Pseudo-first order uptake coefficient developed by Tront and Saunders (2006) for uptake of 2,4,5-trichlorophenol by <i>L. minor</i> (d^{-1})
$k_{20,COD}$	Modified first order removal rate for chemical oxygen demand at 20°C
$k_{T,COD}$	Modified first order removal rate for chemical oxygen demand at constant temperature (d^{-1})
K_{OW}	Octanol-water partitioning coefficient
l	Loss of <i>Lemna minor</i> due to predation and death (d^{-1})
M	Mass of Lemnaceae plants
M_{acc}^{24h}	Theoretical mass accumulation of fluorinated phenols by <i>L. minor</i> at 24-h
N	Nitrogen concentration (mg N/L)
OP	Oxygen production rate ($\mu\text{mol/h-g}$); quantification of plant activity
P	Phosphorus concentration (mg P/L)
r	Maximum growth rate of <i>Lemna minor</i> (d^{-1})
r^2	Square of Pearson's correlation factor (r)
RCF	Root concentration factor
$R(T)$	Relative growth rate of duckweed (d^{-1})
t	Time
T	Temperature ($^{\circ}\text{C}$)
T_{max}	Maximum temperature for Lemnaceae growth ($^{\circ}\text{C}$)
T_{min}	Minimum temperature for Lemnaceae growth ($^{\circ}\text{C}$)
T_{opt}	Optimum temperature for Lemnaceae growth ($^{\circ}\text{C}$)
$TSCF$	Transpiration stream concentration factor

LIST OF ABBREVIATIONS

2-iP	<i>N</i> ⁶ -(3-methylbut-2-enyl)adenine
2-FP	2-Fluorophenol
2-TFMeP	2-Trifluoromethylphenol
2,3-DFP	2,3-Difluorophenol
2,3,6-TCP	2,4,6-Trichlorophenol
2,3,5-TFP	2,3,5-Trifluorophenol
2,3,6-TFP	2,3,6-Trifluorophenol
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4-DCP	2,4-Dichlorophenol
2,4-DFP	2,4-Difluorophenol
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
2,4,5-TCP	2,4,5-Trichlorophenol
2,4,5-TFP	2,4,5-Trifluorophenol
2,4,6-TFP	2,3,6-Trifluorophenol
2,5-DFP	2,5-Difluorophenol
2,6-DFP	2,6-Difluorophenol
3-FP	3-Fluorophenol
3-TFMeP	3-Trifluoromethylphenol
3,5-DFP	3,5-Difluorophenol
4-CP	4-Chlorophenol
4-Cl-IAA	4-Chloroindole-3-acetic acid
4-FP	4-Fluorophenol

4-TFMeP	4-Trifluoromethylphenol
7-TFHOD	2-hydroxy-6-oxo-7,7,7,-trifluorohepta-2,4-dienoate
ANOVA	Analysis of variance
AT	ATP-dependent anion transporter
ATP	Adenosine 5'-triphosphate
B5	Plant tissue culture medium developed by Gamborg et al. (1976)
BA	6-Benzyladenine
BOD	Biological oxygen demand
C Method	Callus induction method for <i>L. gibba</i> developed by Change and Chiu (1976) used for <i>L. minor</i> callus induction
CAS #	Chemical abstract service number
CCWA	Clayton County Water Authority (Jonesboro, GA)
CO	Carbon dioxide
CT	Glutathione-conjugate transporter
d	Day
DAD	Diode array detector
DC	Dissolved carbon
DCPAG	2,4-Dichlorophenyl- β -Dglucopyranosyl-(6 \rightarrow 1)- β -D-apiofuranoside
DDE	1,1- <i>bis</i> -(4-chlorophenyl)-2,2-dichloroethene
DDT	4,4'-(2,2,2-trichloroethane-1,1-diyl)bis(chlorobenzene)
DEET	Meta-N,N-diethyltoluamide
DFP	Difluorophenol
DO	Dissolved oxygen
EC50	Concentration at which 50% inhibition is observed for a given endpoint in a given organism
EPA	U.S. Environmental Protection Agency

ESI	Electrospray ionization for mass spectrometry
Est	Value derived from estimation
Exp	Value derived from experiment
F Method	Callus induction method for <i>L. minor</i> developed by Frick and Morely (2005) used for <i>L. minor</i> callus induction
FC	Fecal coliform bacteria
FP	Monofluorophenol
g ₁	Skewness factor
g ₂	Kurtosis factor
G Method	Callus induction method for <i>L. minor</i> developed by Gunter, et al. (2004) used for <i>L. minor</i> callus induction
GA ₃	Gibberellic acid
GC	Gas chromatography
GT	ATP-dependent glucoside-conjugate
h	Hour
HCB	Hexachlorobenzene
Henry's LC	Henry's law constant
HMX	1,3,5,7-tetranitro-1,3,5,7-tetrazocane
HP	Hydrogen peroxide
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
K _{ow}	Octanol-water partitioning coefficient
K-S	Kolmogorov-Smirnov evaluation of normal distribution
L	Liter
L method	Callus induction method for <i>L. punctata</i> developed by Frick and Morely (2005) used for <i>L. punctata</i> callus induction

LC	Liquid chromatography
LOEC	Lowest concentration at which inhibitory effects are observed
m	Meter
M method	Callus induction method for <i>L. gibba</i> developed by Frick and Morely (2005) used for <i>L. punctata</i> callus induction
m/z	Mass to charge ratio
MS	Plant tissue culture medium developed by Murashige and Skoog (1962)
MS	Mass spectrometer
MW	Molecular weight
N	Nitrogen
NAA	α -Naphthalene acetic acid
NaOCl	Sodium hypochlorite
NH ₃	Ammonia
NMR	Nuclear magnetic resonance
NN	Plant tissue culture medium developed by Nitsch and Nitsch (1969)
O ₂	Oxygen
OM	Dissolved organic matter
OP	Oxygen production rate
P	Phosphorus
PAA	2-Phenylacetic acid
PBDE	Polybrominated-dibenzo ether
PC	Particulate carbon
PCB	Polychlorinated biphenyls
PCE	Perchloroethene
PCP	Pentachlorophenol
pH	Negative log of hydrogen concentration

pK _a	Negative log of acid dissociation constant (K _a)
POP	Persistent organic pollutant, as classified by European Commission
ppt	Parts-per-trillion
Q ₁₀	Temperature coefficient, or ratio by which cyanide removal rates for Chinese elder and weeping willow changed with temperature increase of 10 °C
RDX	1,3,5-trinitroperhydro-1,3,5-triazine
S method	Callus induction method for <i>L. minor</i> developed by Frick and Morely (2005) used for <i>L. minor</i> callus induction
S/A	Sterile or axenic
SH	Plant tissue culture medium developed by Schenk and Hidebrandt (1972)
TC	Tissue culture
TCD	Thermal conductivity detector
TDZ	Thiadiazuron
TFM	3-trifluoromethyl-4-nitrophenol
TFMeP	Trifluoromethylphenol
TFP	Trifluorophenol
TNT	2,4,6-Trinitrotoluene
TSS	Total suspended solids
UDP	Uridine 5'-diphosphate
UV/Vis	Spectra at ultraviolet and visible wavelenghts
VP	Vacuolar peptidase

SUMMARY

Aquatic plants of the family Lemnaceae (e.g., duckweed) actively uptake, metabolize, and sequester pollutants in natural and engineered wetland systems. Numerous interrelated processes contribute to pollutant removal in wetlands; of these processes, interactions between organic pollutants and aquatic plants may be least understood. Research focused on (1) understanding parameters that affected uptake of fluorinated organic pollutants by Lemnaceae, (2) identifying plant processes involved in removal of wastewater-associated organic pollutants by Lemnaceae, and (3) assessing use of Lemnaceae callus cultures in understanding toxicity and metabolism of fluorinated organic pollutants by Lemnaceae. Through active plant uptake, *Lemna minor* rapidly removed 13 fluorinated phenols, with pseudo-first order rate constants of $0.20 \pm 0.04 \text{ d}^{-1}$ to $0.84 \pm 0.07 \text{ d}^{-1}$. Uptake rates depended on substituent type (i.e., trifluoromethyl- vs. fluoro-) and position, with slowest removal rates for di-*ortho*-substituted fluorophenols. Uptake rates decreased with increasing concentrations of fluorinated phenols; assessments of inhibition of plant oxygen production in conjugation with concentration studies indicated that concentration affected uptake rates even when Lemnaceae was not inhibited. Additionally, temperature dependencies of fluorinated phenol uptake by Lemnaceae were well represented by Arrhenius relationships; however, effects of temperature on plant activity were also observed in trends of uptake rates with temperature. Increasing uptake with decreasing concentration and increasing temperature, in addition to effects of plant inhibition, plant activity, and sorption on uptake, strongly emphasized the importance of plant metabolism in uptake of fluorinated phenols by Lemnaceae. Active plant uptake of wastewater-associated organic pollutants affected fate of fluoxetine, triclosan, and 2,4-dichlorophenoxyacetic acid, while passive plant removal processes contributed to fate of four of six wastewater derived organic

pollutants. Consequently, plant-associated processes were important components of fate for over 50% of experimental wastewater-associated organic pollutants. Furthermore, assessments with Lemnaceae callus cultures indicated that callus cultures were generally more susceptible to inhibitory responses to fluorinated phenols than were Lemnaceae plants; however, metabolism of 3-trifluormethylphenol in Lemnaceae callus cultures and plants was similar, indicating that callus cultures may be valuable in plant metabolism studies. Research advances understanding of fate of organic pollutants in wetland systems, and thus has important implications for water quality, ecosystem health, and human health.

CHAPTER 1: INTRODUCTION

Engineering of phytotechnologies presents many opportunities for sustainable development of plant-based approaches to water quality issues. For example, remediation consultants plant plots of hybrid poplar trees to maintain hydraulic control of hazardous pollutant plumes. Farmers frequently install vegetative filter strips to treat field run-off that contains nutrients, pesticides, and fecal bacteria. Municipal water authorities construct wetlands for tertiary treatment and reclamation of wastewater effluents. Even when compared with other phytotechnologies, wetlands epitomize the application of natural systems for addressing water quality issues. Numerous interrelated abiotic and biotic processes contribute to pollutant removal in wetlands, including physical processes like sorption, chemical processes like photodegradation, and biological processes like microbial degradation and plant uptake. Of processes occurring in wetlands, interactions between organic pollutants and aquatic plants may be the least understood in the context of fate and wetland removal of organic pollutants. While research on toxicity of organic pollutants to aquatic plants is plentiful, studies generally rely on standardized testing procedures that do not replicate plant growth as it occurs in wetland systems. Additionally, studies developing predictive relationships for plant uptake and metabolism of organic pollutants have focused on terrestrial plant systems – effects of pollutant properties, pollutant concentrations, and water temperature on uptake of organic pollutants by aquatic plants are largely unknown. Furthermore, while plant tissue cultures have yielded insight into phytometabolism and phytotoxicities of organic pollutants in terrestrial plants, applicability and extrapolation of plant tissue culture results to whole plant systems, especially for aquatic plants and wetlands, is questionable.

Studies using Lemnaceae, or the plant family of duckweeds, present many opportunities for expanding scientific understanding of plant uptake in the context of

wetland systems. Standardized toxicity tests using Lemnaceae can be modified to more closely represent growth in wetland systems. Because of their small size, interactions between organic pollutants and Lemnaceae are easily assessed with entire populations of Lemnaceae, with minimal limitations on transport of organic pollutants to or within Lemnaceae. Additionally, Lemnaceae plants may be ideal for comparisons between plant tissue cultures and whole plants, as their small size and diminished nature (i.e., only possessing fronds and roots) may limit variation in plant tissue culture due to varietal origin of explants.

Scientific understanding of interactions between fluorinated organic pollutants and Lemnaceae is limited. Pollution of waters with fluorinated organic chemicals is increasing because of (1) increased demands on surface waters and resulting reclamation of wastewaters and (2) increased use of fluorination to enhance bioactivity and lifespan of pharmaceuticals and pesticides. By examining plant uptake and phytotoxicities of fluorinated phenols using Lemnaceae populations, a better understanding on effects of fluorination on fate of organic pollutants in wetland systems can be obtained. Furthermore, examining removal of a small group of named organic pollutants (including pharmaceuticals, pesticides, and personal care products) in Lemnaceae systems can establish the relative importance of active plant processes, including uptake, in wetland treatment of wastewaters containing complex organic pollutants. Consequently, the primary aim of the research presented with this thesis is to address the following question:

What is the fate of fluorinated organic pollutants in aquatic plant systems, specifically Lemnaceae and Lemnaceae tissue cultures?

Research consequently examines plant uptake, phytometabolism, and phytotoxicities of fluorinated organic pollutants by two Lemnaceae species (e.g., *Lemna minor* and *Landoltia punctata*). Subsequent research objectives were to:

1. Determine uptake rates for a suite of fluorinated organic pollutants and

identify potential relationships between pollutant properties and uptake.

2. Quantify effects of concentration on uptake, with consideration of parallel concentration effects on plant activity and inhibition.
3. Quantify effects of temperature on uptake of fluorinated organic pollutants by Lemnaceae, with consideration of parallel temperature effects on sorption and plant activity.
4. Identify active and passive plant processes contributing to fate of wastewater-associated organic pollutants in wetland systems, where wastewater derived organic pollutants include pharmaceuticals, pesticides, and personal care products.
5. Evaluate and compare toxicities and metabolism of fluorinated organic pollutants in whole Lemnaceae and Lemnaceae callus cultures.

CHAPTER 2: LITERATURE REVIEW

Wetlands for treatment and reclamation of wastewaters

Constructed wetlands are extensively used in the United States and worldwide for treatment and reclamation of municipal and agricultural wastewaters. As compared to conventional treatment systems, constructed treatment wetlands are substantially more cost effective, with significantly lower lifetime costs and frequently lower capital costs (Interstate Technology and Regulatory Council Wetlands Team 2003), although direct comparison on levels of removal of a broad array of pollutants are complex. Constructed treatment wetlands can tolerate fluctuations in flow and pollutant concentrations, provide aquatic habitats, and enhance aesthetics associated with wastewater treatment. Abiotic and biological processes occur concurrently in wetlands to remove pollutants, including total suspended solids (TSS), biochemical oxygen demand (BOD), nutrients (N and P), organic pollutants, and inorganic pollutants. Abiotic processes include settling and sedimentation, sorption to soils and plants, chemical oxidation and reduction with subsequent precipitation, photodegradation or oxidation, and volatilization. Biological removal processes, including aerobic and anaerobic biodegradation, plant uptake and sequestration, phytoaccumulation, phytostabilization, phytodegradation, rhizodegradation, and phytovolatilization, also play significant roles in treatment of wastewaters in constructed treatment wetlands (Interstate Technology and Regulatory Council Wetlands Team 2003). Removal processes and potential sinks for organic pollutants in wetland systems are depicted in Figure 2.1. Typical removals of wastewater constituents in domestic wastewater systems by 21 constructed treatment wetlands in North America are provided in Table 2.1.

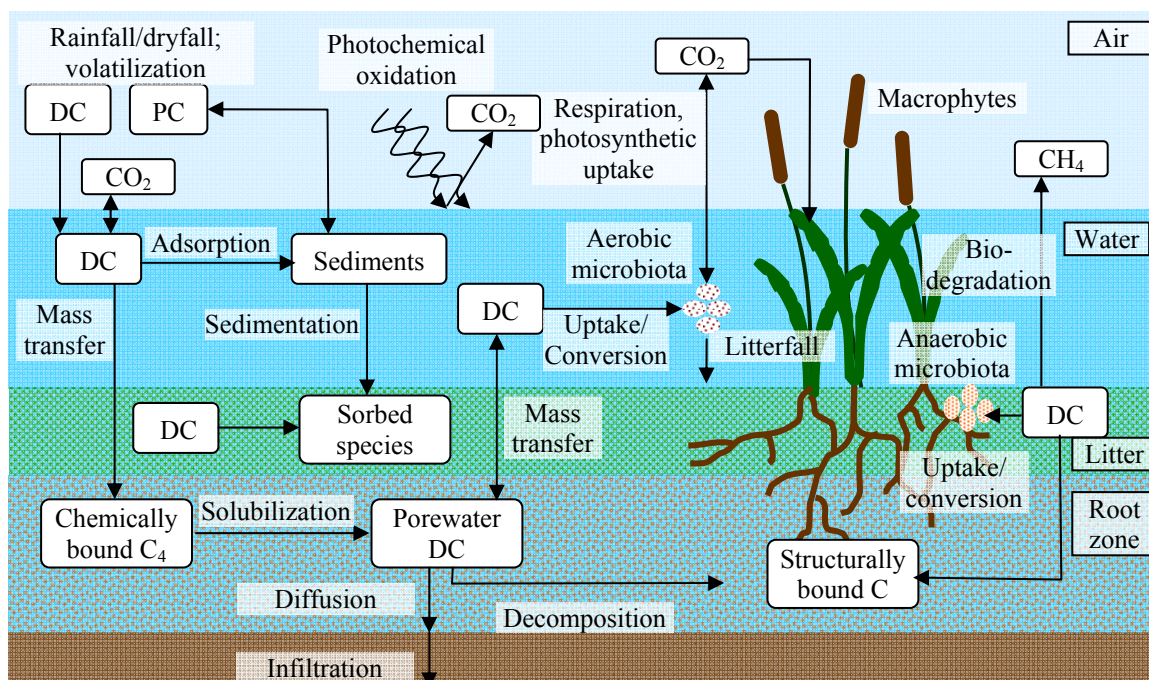


Figure 2.1. Mechanisms for organic carbon removal in wetland systems. Abbreviations indicate dissolved carbon (DC) and particulate carbon (PC). Figure adapted from Technical and Regulatory Guidance for Constructed Treatment Wetlands (2003).

Table 2.1. Removal of wastewater constituents by 21 constructed treatment wetlands in North America. Data from (Kadlec 2003).

Wastewater constituent	Maximum removal	Minimum removal	Mean removal	Median removal
Total suspended solids (TSS)	93%	27%	65%	67%
Biological oxygen demand (BOD ₅)	87%	17%	58%	61%
Ammonia (NH ₃)	99%	-33%	50%	61%
Phosphorus (P)	99%	7%	49%	48%
Fecal coliforms (FC)	99.8%	-179%	20%	77%

Free-surface wetlands or ponds covered with Lemnaceae (i.e., duckweed) are effective systems for treatment and reclamation of wastewaters. Model Lemnaceae systems removed 120 – 590 mg N/m²-d (73 – 97% of initial N concentrations) and 14 – 74 mg P/m²-d (63 – 99% of initial P concentrations), indicating that wetland systems effectively remove nutrients from wastewaters (Korner and Vermaat 1998). Plant uptake accounted for 30 – 47% of nitrogen removal and <52% of phosphorus removal; additional abiotic and biotic processes, including volatilization, microbial denitrification/nitrification, and uptake by microorganisms and algae, accounted for further removal. Similar studies have also demonstrated high N and P removal rates (i.e., 94% and 77% respectively) for *Lemna gibba* systems exposed to wastewaters (Vermaat and Hanif 1998).

Proper management and application is crucial to address potential disadvantages of wetland treatment of wastewaters. Management of increased mosquito populations near free-water constructed treatment wetlands is frequently required. Introduction of fecal coliforms from wildlife is another concern associated with wetlands for reclamation. Almost doubling of fecal coliforms has been observed wetland systems (Table 2.1). Removal of fecal coliforms is frequently in conflict with the goal of increasing wildlife habitat through construction of treatment wetlands. Additionally, further physicochemical treatment for phosphorus, including slag filters or chemical precipitation, may be required for wetland effluents to meet regulatory discharge standards of 0.3 mg P/L (Interstate Technology and Regulatory Council Wetlands Team 2003). With respect to organic pollutants, introduction of pollutants to aquatic ecosystems is a major concern. Fish, collected from a wetland maintained by secondary-treated wastewater effluents, accumulated hydrophobic organic pollutants, included DDE and *trans*-nonachlor (Barber et al. 2006). Consequently, increased understanding of the fate of organic pollutants in wetland systems is crucial to further application and development of constructed treatment wetland technologies.

Reports of wetland removals of organic pollutants are emerging as society becomes more concerned about organic pollutants, including pesticides, phenols, pharmaceuticals, and personal care products. However, removal is typically process and pollutant specific and should be evaluated on a case-by-case basis. Constructed treatment wetlands were effective at removing estrogenic pollutants and steroids from swine wastewaters (Shappell et al. 2007). Wetlands removed 83 to 93% of estrogenic activity and effluent concentrations of estrogenic pollutants were less than concentrations known to impact fish populations (i.e., <37 ng/L). Pilot-scale, subsurface-flow wetlands with *Phragmites* (common reed) removed substantial quantities of ibuprofen and carbamazepine (i.e., up to 81% and 26% respectively); however, removal of clofibric acid was negligible (Matamoros et al. 2005). Consequently, while wetlands hold potential for treatment of wastewaters containing organic pollutants, much research is needed to define and understand removal processes for organic pollutants in wetlands.

As removal in wetland systems depends on many related and interconnected processes, wetland efficiency can depend on many parameters. Influent concentrations, influent flow rates, plant species, wetland surface area and depth, and wetland hydraulics can all affect wetland efficiencies. Relationships between operational parameters and removal in wetland systems are also complex and occasionally counterintuitive. Additionally, some environmental parameters not only directly affect wetland efficiency, but also affect and depend on growth of wetland plants. One specific parameter that demonstrates the complicated relationships between operational parameters and overall wetland removal of wastewater constituents is temperature; relationships between temperature and wetland removal of wastewater constituents are discussed further to illustrate complexity associated with predicting effects of operational parameters on wetland removals.

Wetland treatment efficiency is greatly affected by season, with lower efficiencies frequently observed in winters. Temperature affects many biological processes in

wetlands, including microbial removal of organic matter, microbial ammonification, nitrification, and denitrification, and plant uptake of nitrogen and phosphorus (Kadlec and Reddy 2001). Consequently, predicting temperature effects on removal of wastewater constituents in wetlands is not straightforward. Removal of biochemical oxygen demand (BOD) and total suspended solids by wetlands demonstrated little to no dependence on temperature (Kadlec and Reddy 2001). However, greenhouse studies indicated temperature affects chemical oxygen demand (COD) removal in wetlands. COD removal in wetland columns depended on temperature and was well represented using the Arrhenius relationship:

$$k_{T,COD} = k_{20,COD} \theta^{T-20} \quad \text{Equation 2.1}$$

where $k_{T,COD}$ is COD removal rate constant at temperature T (d^{-1}), $k_{20,COD}$ is COD removal rate constant at 20°C, θ is dimensionless temperature coefficient, and T is temperature (°C) (Stein et al. 2006). Removal decreased with increasing temperature and fitted Arrhenius parameters depended on plant species; for example, θ values were 0.945, 0.957, and 0.953 for beaked sedge, hard bulrush, and cattail. Authors suggest that decreasing COD removal with increasing temperature resulted from increase in unfavorable redox conditions (e.g., anaerobic zones) and consequent decrease in microbial degradation of COD (Stein et al. 2006). Additionally, temperature effects on removal of dissolved organic matter (OM) in subsurface flow wetland mesocosms also depended on plant species. In unplanted controls and broadleaf cattail mesocosms, removal of OM decreased with decreasing temperature (4°C to 24°C), while, in beaked sedge and hard bulrush mesocosms, removal of OM was enhanced at low temperatures (Allen et al. 2002). Consequently, temperature effects on wetland removal of three similar wastewater constituents (i.e., BOD, COD, OM) indicated that wetland removal was constant, decreased, or increased with increasing temperature. Removal of organic pollutants may also be temperature dependent. Removal of triclosan in Tres Rios Demonstration Constructed Wetlands (near Phoenix, AZ) was negligible (i.e., -6%) in the

summer, but was enhanced, with removal as great as 29%, during winter months (Barber et al. 2006). However, research on additional organic pollutants in controlled systems is needed to fully understand role of temperature in wetland removal of organic pollutants.

Constructed treatment wetlands consistently demonstrate cost-effective treatment of traditional wastewater constituents, including COD, TSS, and nutrients. Wetlands also have potential to remove organic pollutants that may be present in wastewaters.

Relationships between operational parameters and wetland removal of wastewater constituent are generally complex and frequently depend on plant species present in wetlands. However, roles of plant processes in removal of wastewater constituents, especially organic pollutants, are rarely delineated in many laboratory and most field studies. Therefore, increased understanding of plant-associated removal processes in wetlands, particularly for organic pollutants, is needed; insight provided from controlled systems utilizing model plant species will be invaluable in delineating the role of plants in wetland removal of organic pollutants.

The duckweed family, Lemnaceae

Duckweed is the common name for a family of floating aquatic plants known scientifically as Lemnaceae. Lemnaceae species are widely distributed and are commonly found in quiet freshwater habitats. Lemnaceae consists of two subfamilies: Lemnoideae with 17 species and Wolffioideae with 21 species, as shown in Figure 2.2 (Les et al. 2002). Lemnaceae species are distinguished by their diminutive nature and miniaturization of organs. Shoots are not differentiated into “leaves” and “stems”, but instead are reduced to a small green thallus or “frond”. Lemnaceae species reproduce primarily through asexual budding, and have rapid growth rates. For example, during an average lifespan of 31.3 ± 1.1 days, a *Lemna minor* mother frond can produce 14.0 ± 0.5 daughter fronds at a production rate of 0.45 ± 0.02 fronds per day (Lemon et al. 2001). *Landoltia punctata* possesses a shorter lifespan of 12.1 ± 1.1 d and a slower production

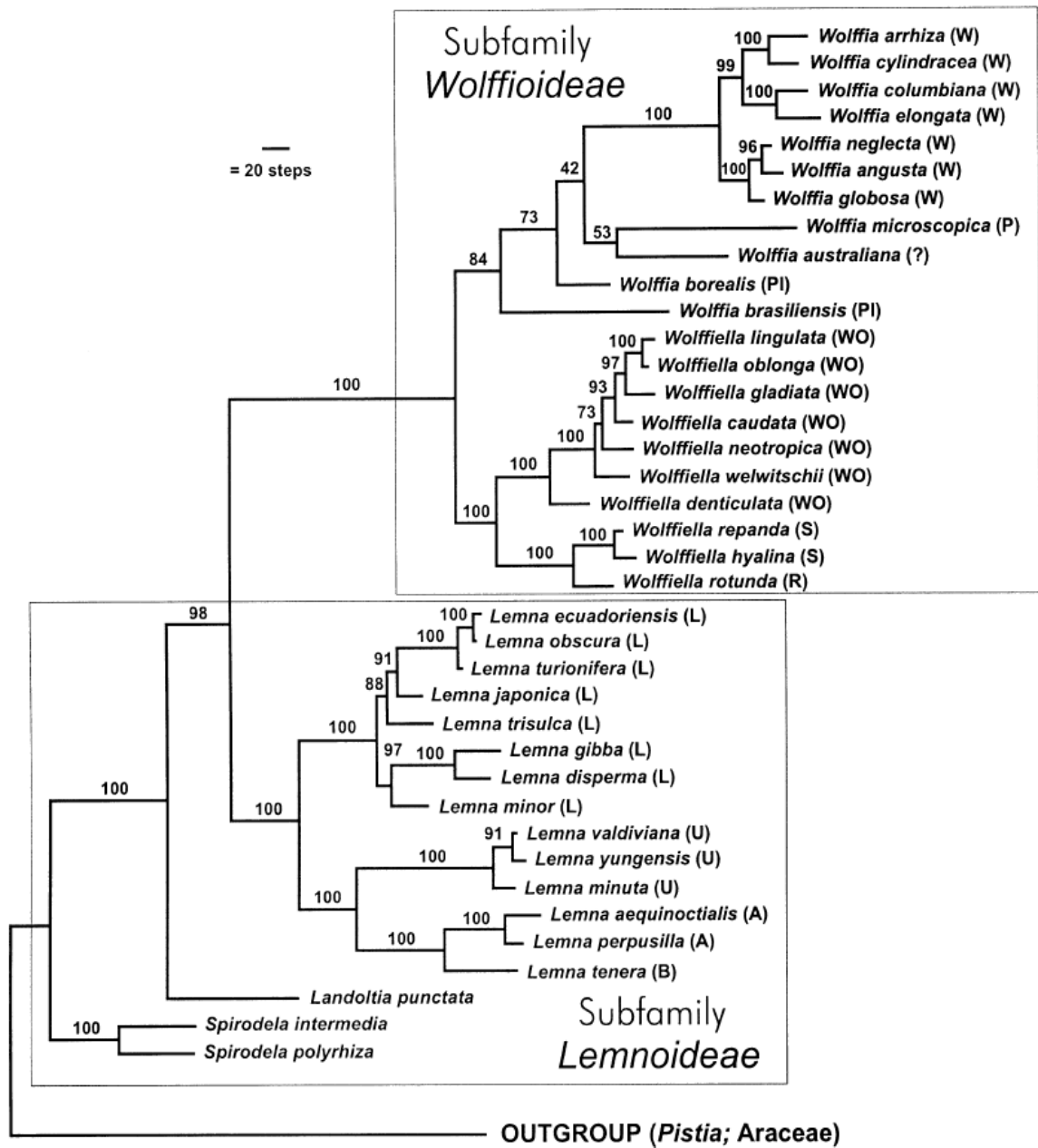


Figure 2.2. Cladogram of Lemnaceae species based on morphological, flavonoid, allozyme, and DNA sequence data. Bootstrap support for nodes is indicated above branches. Figure from (Les et al. 2002).

rate of 0.08 ± 0.02 fronds per day (Lemon et al. 2001).

As flowering and fruiting are rarely observed in Lemnaceae, dichotomous keys based on vegetative characteristics are useful in classifying species (Armstrong 2001). Two species, *L. minor* and *L. punctata* were identified as common species of duckweeds growing in wetlands and quiescent waters near Atlanta, GA. *L. minor* possesses one root and a 2 to 4 mm green frond with smooth dorsal surface and absence of reddish anthocyanin on the ventral surface. In contrast, *L. punctata* is characterized by 1 - 5 roots and slightly larger fronds (3 to 5 mm in length) with a dark green dorsal surface and reddish-purple ventral surface. It is important to note that *L. punctata* is a relatively new classification (Les and Crawford 1999), and this species was previously referred to as *Spirodela punctata*. A dichotomous key for identification of Lemnaceae species is provided by Armstrong (2001). In addition to the dominance of Lemnaceae populations by these two species, *Spirodela polyrhiza*, *Lemna obscura*, non-gibbous *Lemna gibba*, and *Wolffia* species occasionally grow in Clayton County Water Authority constructed wetlands.

Parameters affecting Lemnaceae growth and reproduction

Lemnaceae growth depends on many water quality parameters, including nutrients and pH; additionally, environmental parameters, including temperature can affect Lemnaceae growth. Lemnaceae species, including *L. punctata*, *L. gibba*, *L. minor*, *L. obscura*, and *Lemna aequinoctialis*, can reproduce and uptake nitrogen and phosphorus at the relatively high concentrations of nitrogen and phosphorous associated with swine waste lagoons. For example, Lemnaceae rapidly grew, at a rate of $1.33 \text{ g/m}^2\text{-h}$, in simulated wastewater with 240 mg N/L ammonium and 31.0 mg P/L phosphate (pH~7); corresponding nutrient uptake rates were 0.955 mg N/L-h for ammonium and 0.129 mg P/L-h for phosphate (Cheng et al. 2002). *L. minor* growth is also affected by low total nitrogen and phosphorous concentrations. Nutrient limitations were described using

Monod-like functions, with half-saturation values for nitrogen and phosphorus of 0.04 mg N/L and 0.05 mg P/L (Luond 1980).

Ammonium concentration, in conjunction with pH, affects growth of *S. polyrhiza* (Caicedo et al. 2000). Maximum growth was observed when total ammonium concentrations were 3.5 to 20 mg/L N. Growth was inhibited with increasing pH and increasing ammonium concentrations at pH 5 to 8 and ammonium concentrations of 3.5 to 100 mg/L NH₄-N. Unhealthy, yellowish growth of *S. polyrhiza* was observed at pH<6, while frond death was observed at pH>8; the optimal pH for *S. polyrhiza* growth was approximately 7 (Caicedo et al. 2000). In contrast, *L. gibba* has been reported to grow in pH values ranging from pH 3.5 to 10.5 (Cleuvers and Ratte 2002).

Temperature effects on Lemnaceae growth and reproduction are well studied and have been described using multiple equations. Temperature limitations on Lemnaceae growth were first described as linear between a minimum temperature of 5 °C and optimum temperature of 26 °C (Landolt 1986). van der Heide et al (2005) subsequently proposed the following description of temperature-dependent growth of Lemnaceae:

$$R(T) = cT(T - T_{\min})(T_{\max} - T) \quad \text{Equation 2.2}$$

$$T_{\text{opt}} = \frac{1}{3} \left(T_{\max} + T_{\min} + \sqrt{T_{\max}^2 - T_{\min}T_{\max} + T_{\min}^2} \right) \quad \text{Equation 2.3}$$

where R is relative growth rate (d⁻¹), c is an empirical scaling constant, T is temperature, and subscripts indicate optimum (*opt*), maximum (*max*), and minimum (*min*) temperatures. Fitted values ($r^2 = 0.95$) for temperature dependent growth, based on equations 2.2 and 2.3, were $T_{\min} = 6.2 \pm 0.6$ °C, $T_{\text{opt}} = 25.7$ °C, $T_{\max} = 36.8 \pm 0.2$ °C.

Temperature dependency of Lemnaceae growth is complicated by accurate field measurement of local water temperatures under dense Lemnaceae growth. Vertical temperature gradients in Lemnaceae ponds resulted in 1.9°C difference between

temperatures at pond surface and at 64 cm depth during the day time; temperature gradients were negligible at nighttime (Caicedo et al. 2002). Temperature gradients were less than those typically observed in conventional stabilization ponds, suggesting that Lemnaceae mats play an important role in decreasing local surface water temperatures via absorption and reflection of sunlight energy.

Growth of Lemnaceae also exhibits an Allee effect, or inverse density dependence. In large-scale laboratory growth studies, specific growth (% mass increase per day) decreased hyperbolically with increase in plant density (g dry mass/ m²); optimal stock densities, with weekly harvests of 6 to 7 g fresh mass/m²d, were 20 g/m² (Debusk and Ryther 1981). Growth rate of *L. minor* in natural ditches was accurately described using a Monod-type limitation function (Driever et al. 2005):

$$\frac{dB}{dt} = B \cdot r \cdot \left(\frac{T - T_{\min}}{T_{\text{opt}} - T_{\min}} \right) \left(\frac{N}{N + h_N} \right) \left(\frac{P}{P + h_P} \right) \left(\frac{h_B}{B + h_b} \right) - l \cdot B \quad \text{Equation 2.4}$$

where B was biomass surface coverage (g dry mass/m²), r was maximum growth rate (d⁻¹), and l was loss (d⁻¹). Biomass production was dependent on air temperature (T), surface coverage (B), nitrogen concentration (N) and phosphorus concentration (P). The parameters h_N , h_P , and h_B represented half-saturation values for nitrogen, phosphorus, and surface coverage limitations on growth and had values of 0.04 mg/L N (Luond 1980), 0.05 mg/L P (Luond 1980), and 26 g dry mass/m² (Driever et al. 2005), respectively. The minimum and optimal temperatures (T_{\min} , T_{opt}) were 5°C and 26°C, respectively (Landolt 1986; Landolt and Kandeler 1987). Fitted values for r and l were 0.41 d⁻¹ and 0.05 d⁻¹, respectively. Maximum observed density increased with increasing air temperature and lower growth rates with high plant density were attributed to lower local temperatures in water columns directly beneath Lemnaceae mats (Driever et al. 2005). Consequently, Lemnaceae growth and water temperature were co-dependent, emphasizing the

complexity of relationships between aquatic plants and environmental parameters in wetlands.

Phytotoxicity assessments using Lemnaceae species

Lemnaceae species are commonly used in experimental studies because of their simple morphology and rapid growth. Standardized toxicity testing methods for using species from the Lemnoideae subfamily of Lemnaceae have been adopted by American Society for Testing and Materials (ASTM 1997), U.S. Environmental Protection Agency (EPA 1996), and American Public Health Association, American Water Works Association, and Water Environment Federation (APHA et al. 1998b). Of Lemnaceae species, *L. minor* or *L. gibba* are most frequently utilized to assess physiological responses to chemicals. Lemnaceae species have been used to determine toxicities of metals (Teisseire et al. 1998; Frankart et al. 2002), pharmaceuticals (Pro et al. 2003; Brain et al. 2004a; Pomati et al. 2004), pesticides (Verdisson et al. 2001; Geoffroy et al. 2004), and halogenated organic pollutants (Huber et al. 1982; Hanson et al. 2002; Hanson and Solomon 2004). Table 2.2 lists examples of experimental toxicity assessments using Lemnaceae species.

Toxicity tests with Lemnaceae utilize a variety of experimental procedures and analytical techniques. Generally, standardized tests use a low number (e.g., 10 – 20) of plant fronds in test vessels at surface coverages that are extremely low, especially when compared to surface coverages representative of natural waters, wetlands, or oxidation pond systems. Instead, a maximum of 12 fronds (APHA et al. 1998b) or 16 fronds (ASTM 1997) are exposed to aqueous pollutants for 7 d. After exposure, frond health or growth is assessed by one or multiple endpoints, including frond number, dry biomass, and root length. Biochemical end-point assessments include oxygen production (Tront and Saunders 2006), photosynthetic oxygen-evolution (Huber et al. 1982; Geoffroy et al. 2004), and chlorophyll fluorescence {Huber, 1982 #389; Hulsen, 2002 #357; Brain, 2004 #126}.

Table 2.2. Examples of toxicity assessments using Lemnaceae species.

Organic pollutants	Lemnaceae species	Endpoints	Time (d)	Reference
Pesticides				
Flumioxazin	<i>L. minor</i>	Pigment, chlorophyll fluorescence, antioxidative enzyme activities, growth	2	(Geoffroy et al. 2004)
Pentachlorophenol	<i>L. minor</i>	Oxygen production, chlorophyll content, glutamate dehydrogenase activity, alanine aminotransferase activity, structural changes in chloroplast membranes	2.5	(Huber et al. 1982)
Procymidone, fludioxonil, and pyrimethanil	<i>L. minor</i>	Relative growth rate, chlorophyll content	6	(Verdisson et al. 2001)
Pharmaceuticals				
Antimicrobial pharmaceuticals	<i>L. minor</i>	Number of green fronds, frond number, number of chlorotic fronds, number of colonies, fresh biomass, dry biomass, daily growth rate	7	(Pro et al. 2003)
Pharmaceuticals (25)	<i>L. gibba</i>	Wet biomass, frond number, chlorophyll content, carotenoids	7	(Brain et al. 2004a)
Pharmaceuticals (8)	<i>L. gibba</i>	Frond number, growth rate, chlorophyll content, carotenoids	14	(Brain et al. 2004b)
Fluoroquinolone antibiotics (7)	<i>L. minor</i>	Reproduction rate	7	(Robinson et al. 2005)
Tylosin	<i>L. gibba</i>	Frond number, growth rate, chlorophyll content, carotenoids	7	(Brain et al. 2005)
Flumequine	<i>L. minor</i>	Fresh mass, chlorophyll content	35	(Cascone et al. 2004)

Table 2.2. (continued)

Organic pollutants	Lemnaceae species	Endpoints	Time (d)	Reference
Industrial effluents				
Industrial effluents (8)	<i>L. minor</i>	Mortality, chlorosis, necrosis, lesions, loss of buoyancy	2, 5	(Wang and Williams 1988)
Industrial effluents (8)	<i>L. minor</i>	Net frond increase	4	(Wang and Williams 1990)
Other organic pollutants				
Polyaromatic hydrocarbons (13)	<i>L. gibba</i>	Chlorophyll <i>a</i> Growth rate	7	(Mallakin et al. 2002)
Phenol and chlorophenols	<i>L. gibba</i>	Growth rate	7	(Sharma et al. 1997)
Triphenyltin	<i>L. minor</i>	Chlorophyll Sugar content Chloroplast activity Peroxidase activity	8	(Song and Huang 2001)

Choice of endpoint is highly dependent on pollutant mode of toxicity and research laboratory preferences. For example, number of green fronds at 168 h was the best indicator, in terms of relevance, cost, and effectiveness, for assessing toxicity for antimicrobial pharmaceuticals when compared to total number of fronds, number of chlorotic fronds, number of colonies, fresh biomass, dry biomass, and daily growth rate (Pro et al. 2003). However, non-photochemical quenching of chlorophyll fluorescence was the most sensitive 48-h endpoint for *L. minor* exposed to flumioxazin; relative sensitivities at 48-h for many endpoints were: *in vivo* chlorophyll fluorescence and oxygen emission > catalase activity and glutathione reductase activity > pigment > ascorbate peroxidase activity > growth (Geoffroy et al. 2004).

Fate of Organic Pollutants in Aquatic Plant Systems

Plant uptake of organic pollutants

Despite the crucial role aquatic plants play in removing many organic pollutants from surface water supplies (Bhadra et al. 1999; Crum et al. 1999; Gao et al. 2000; McCutcheon and Schnoor 2003), active plant-driven accumulation and metabolism of organic pollutants is often neglected in consideration of environmental fate. However, plants may serve as reservoirs for organic pollutants and as conduits for introducing organic pollutants into animal and human food supplies. Through sorption, diffusion, partitioning, and translocation, aqueous-phase pollutants accumulate in plants. For example, black willow (*Salix nigra*) and bulrush (*Scirpus olneyi*) removed approximately 5% of desorption-resistant ^{14}C -labeled phenanthrene and chlorobenzene from sediments through root sorption, uptake, and translocation (Gomez-Hermosillo et al. 2006).

Uptake of organic pollutants, especially pesticides, has been extensively studied in terrestrial plants. Uptake of *O*-methylcarbamoyloximes and substituted phenylureas by barley roots indicated that uptake depended on hydrophobicity of organic pollutant, as quantified by octanol-water partitioning coefficient (K_{OW}) (Briggs et al. 1982). Concentration of organic pollutant accumulated in barley roots was predicted by the equation:

$$\log RCF = 0.77 \log K_{OW} - 1.52 \quad \text{Equation 2.5}$$

where RCF is root concentration factor and was defined by concentration in roots divided by concentration in external solution. Likewise, concentration of organic pollutant translocated from roots to shoots, as described by transpiration stream concentration factor (TSCF; concentration in transpiration stream divided by concentration in external solution), was related to hydrophobicity:

$$\log TSCF = 0.784 \exp - \frac{(\log K_{OW} - 1.78)^2}{2.44} \quad \text{Equation 2.6}$$

The Gaussian relationship between TSCF and $\log K_{OW}$ (Equation 2.6) is believed to describe an optimum hydrophobicity for translocation. At $\log K_{OW} < 1.8$, organic pollutants may not partition through lipid membranes in epidermal root cells, while, at $\log K_{OW} > 1.8$, organic pollutants may not enter xylem cells for translocation. While translocation biochemistry differs among plant species, a similar Gaussian relationship has been developed for uptake of organic pollutants by hybrid poplar trees (Burken and Schnoor 1998):

$$\log TSCF = 0.756 \exp - \frac{(\log K_{OW} - 2.50)^2}{2.58} \quad \text{Equation 2.7}$$

indicating that hydrophobicity may play an important role in uptake of organic pollutants by most species of terrestrial plants.

Models have been developed to quantitatively explain uptake of organic pollutants by plants, including models by Paterson et al. (1990), Trapp and Matthies (1995), and Chiou et al. (2001). Research typically focused on terrestrial plants and an extensive inter-comparison of models was conducted (Collins and Fryer 2003).

Processes that were incorporated into reviewed models included:

- Passive uptake from soil (8 of 9 models),
- Deflection at endodermis (1 of 9 models),
- Transpiration stream flow (6 of 9 models),
- Assimilation stream flow (2 of 9 models),
- Partitioning into plant tissues (9 of 9 models),
- Leaf-air diffusive transfer (6 of 9 models),
- Particle deposition (1 of 9 models),

- Soil-air diffusive transfer (2 of 9 models),
- Plant metabolism (4 of 9 models), and
- Growth diffusion (4 of 9 models).

In comparing experimental results for uptake of 19 organic pollutants by 7 plant species, Collins and Fryer (2003) found that dynamic models were well suited for acute exposures, while equilibrium and steady-state models were sufficient for chronic exposures.

Aquatic and terrestrial plants may differ substantially in uptake of organic pollutants. Processes included in terrestrial uptake models, like passive uptake from soils, soil-air diffusive transfer, and leaf-air diffuse transfer, are likely negligible in uptake of organic pollutants by free-floating aquatic plants. Additionally, partitioning into plant tissues from water is not limited to root systems, but may occur in all plant tissues. A strong indicator of uptake of organic pollutants by *L. minor* fronds is that uptake of chlorophenols by *L. minor* was not impacted by removal of roots (Day 2002). Additionally, studies with *L. minor* indicate that hydrophobicity may not be a descriptive parameter of organic pollutant uptake by floating plants. Uptake of halogenated phenols by *L. minor*, as defined by pseudo-first-order rate coefficients, was independent of pollutant K_{OW} (Tront et al. 2007). Decreased importance of translocation in Lemnaceae and other floating aquatic plants, combined with uptake of organic pollutants through fronds in addition to roots, may account for lessened impact of hydrophobicity on uptake. Independence of uptake of halogenated phenols by *L. minor* on $\log K_{OW}$ may also indicate that partitioning of halogenated phenols does not limit uptake.

Uptake of 2,4,5-trichlorophenol (2,4,5-TCP) by *L. minor* has been previously described as a sequential series of partitioning, metabolism, and sequestration and has been modeled with the following first-order exponential decay relationship (Tront and Saunders 2006):

$$\frac{dC_{aq}}{dt} = k^* \alpha \beta C_{aq} \quad \text{Equation 2.8}$$

where C_{aq} is aqueous concentration (μM), t is time (d), k^* is uptake rate coefficient (normalized to α and β), α is plant activity ($\text{mmol O}_2/\text{h}$), and β is inhibition of *L. minor* due to pollutant exposure. Pollutant speciation substantially influenced uptake and was reflected in k^* term; results indicated that only the neutral, or protonated, form of 2,4,5-TCP partitioned into *L. minor*. Inhibition was defined as:

$$\beta = 1 - \frac{a}{1 + \exp\left(-\left(\frac{C_i - C_{50}}{b}\right)\right)} \quad \text{Equation 2.9}$$

where C_i was internal concentration of organic pollutant, C_{50} is concentration of internal pollutant at which *L. minor* was 50% inhibited, and a and b are fitting parameters.

Few studies have examined the role of plants in context with overall environmental fate of organic pollutants. When tetrachloroguaiacol was introduced into “ecotoxicity” reactors containing sediments, water, mussels (*Anodonta piscinalis*), fish (*Carassius carassius*), and Lemnaceae, approximately 37.2% removal was observed (Mikkelsen et al. 1995). Sediments, mussels, fish, and Lemnaceae served as reservoirs for tetrachloroguaiacol, with sediments and fish accounting for most of the detected, untransformed tetrachloroguaiacol (68.5% and 29.8%, respectively). Almost negligible amounts of tetrachloroguaiacol were detected in mussels and Lemnaceae (1.3% and 0.4%, respectively). Degradation products, primarily lesser chlorinated chloroguaiacols or chloroveratroles, also accumulated in sediments; over 87% of detected degradation products were found in sediments. Accumulation of degradation products was substantially reduced in mussels (4.2%), fish (7.4%), and plants (0.5%). However, over 17% of tetrachloroguaiacol was not found, either in parent form or as degradation

products, and experimental design did not examine nor account for conjugation products of tetrachloroguaiacol, which may result from animal or plant metabolism of tetrachloroguaiacol (Mikkelsen et al. 1995).

Phytometabolism of organic pollutants

Continued uptake of organic pollutants depends on plant metabolism of internal organic pollutants – a serial chain of biochemical reactions that can include transformation of parent pollutants, conjugation of metabolites with macromolecules, and incorporation of conjugated products into cell walls and vacuoles. Figure 2.3 depicts metabolism of organic pollutants by plant cells. Plant metabolism is considered similar to mammalian liver metabolism of exogenous organic chemicals and has consequently been deemed “green liver” metabolism (Sandermann 1994). Three phases of metabolism have been defined: Phase I consists of transformation of organic pollutants, Phase II consists of conjugation of parent pollutant and Phase I metabolites, and Phase III consists of sequestration or compartmentalization of Phase II metabolites (Sandermann 1992; Coleman et al. 1997; McCutcheon and Schnoor 2003).

Phase I metabolism includes transformation of organic pollutants via hydroxylation and oxidation. Cytochrome P-450 is crucial in Phase I metabolism; however, necessity of Phase I transformations depends on organic pollutant structure and availability of functional groups suited for Phase II metabolism. For example, metabolism of chlorophenols proceeds directly to Phase II conjugation (Barber et al. 1995; Day and Saunders 2004). Despite ability of plants to bypass Phase I metabolism of chlorophenols, plants exposed to chlorophenols increased production of intracellular and extracellular enzymes associated with Phase I metabolism. When axenic *L. punctata* was exposed to 2,4,6-trichlorophenol, peroxidases capable of oxidative dechlorination of chlorinated phenols in the presence of hydrogen peroxide were released into aqueous medium (Jansen et al. 2004). This detoxification mechanism was specific to introduction

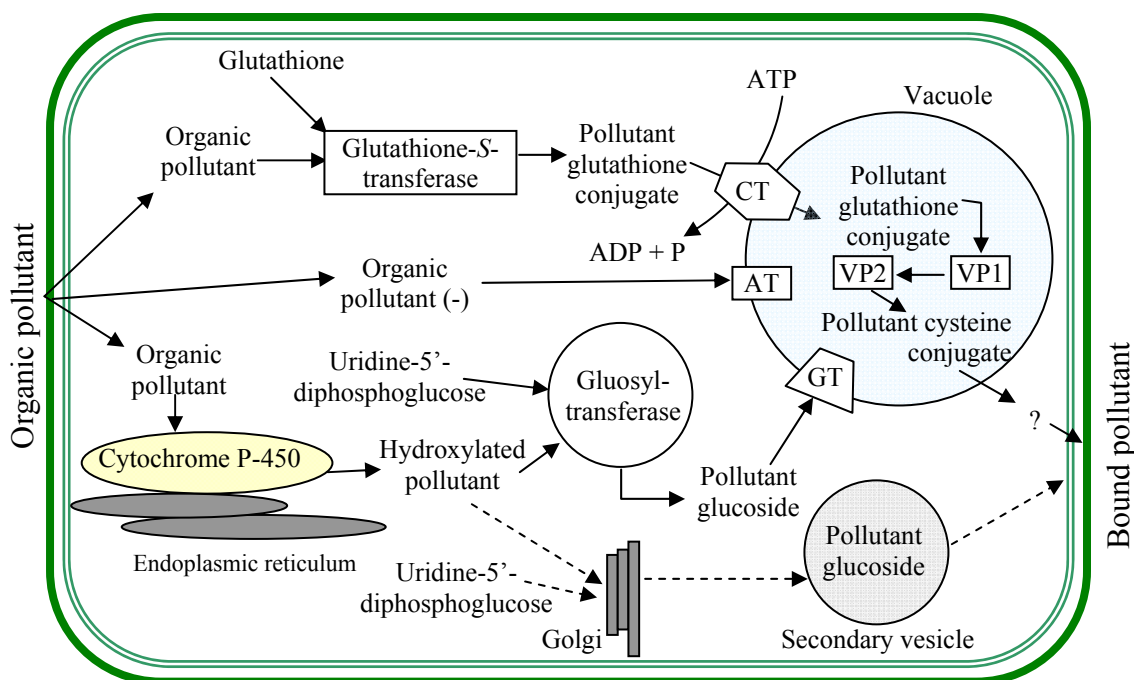


Figure 2.3. Depiction of plant metabolism of organic pollutants. Broken arrows represent proposed pathway for glucosylation in Golgi. CT is glutathione-conjugate transporter, AT is ATP-dependent anion transporter, GT is ATP-dependent glucoside-conjugate transporter, and VP is vacuolar peptidase. Taken from Coleman et al. (1997).

of phytotoxic chlorophenols, required healthy fronds, and was not induced by abiotic stress factors. Interestingly, *L. minor* uptake of 2,4,6-trichlorophenol was negligible, suggesting that steric hindrance of glucosyltransferases may interfere with Phase II metabolism of 2,4,6-trichlorophenol, especially when compared to other chlorinated phenols that were rapidly uptaken by *L. minor*. Therefore, increased Phase I metabolism of 2,4,6-trichlorophenol may result from enzymatic limitations of Phase II metabolism.

Phase II metabolism of organic pollutants by plants relies upon conjugation of Phase I metabolites or parent organic pollutants. Two primary conjugation pathways are prevalent in plant detoxification of organic pollutants: the glycosylation pathway and glutathione pathway (Sandermann 2004b). The *Arabidopsis* Genome project has identified over 480 enzymes involved in plant conjugation of organic pollutants; greater than 110 UDP-glucose-dependent transferases have been identified. It has been

concluded that plant cells contain multiple, overlapping isoenzymes with various substrate specificities (Schaffner et al. 2002). Conjugation and sequestration of pollutants into plant biomass eliminates phytotoxicity associated with parent pollutant. Plant conjugation of herbicides produces products that are generally inactive toward the initial target site, are more hydrophilic and thus less mobile, and that may undergo further plant processing, including secondary conjugation and compartmentalization (Kreuz et al. 1996). Increase in enzymes capable of conjugation is observed quickly upon exposure of plants to phytotoxic compounds; glutathione *S*-transferase was induced in *L. minor* after 6 h exposure to the fungicide folpet (Teisseire and Vernet 2001).

Lemnaceae species are valuable in researching the removal of organic pollutants by aquatic plants. At full surface coverage, *L. minor* quickly processes halogenated aromatic compounds; the aqueous-phase half-life for removal of 8 μ M 2,4,5-TCP by *L. minor* (10 g fresh mass/L) was 16 to 22 h (Tront et al. 2007). Primary metabolism of 2,4-dichlorophenol (2,4-DCP) and 2,4,5-TCP proceeds primarily through glycosidation in *L. minor*, yielding chlorinated malonyl and apiose conjugates that were incorporated into vacuoles and cell walls, as shown in Figure 2.4 (Day and Saunders 2004). Assimilation of 2,4-DCP and 2,4,5-TCP following conjugation with carbohydrates by *L. minor* concurs with published data. Pentachlorophenol, 2,4,5-TCP, 2,4-dichlorophenoxyacetic acid, and phenol are all reported to undergo glycosylation, particularly glucosylation, in plants (Frear et al. 1983a; Casterline Jr. et al. 1985; Barber et al. 1995; Sharma et al. 1997; Laurent et al. 2000). 2,4,5-TCP is a superior active substrate for many glycosyltransferases (Schaffner et al. 2002; Sandermann 2004b). Multiple UDP-glucose dependent glycosyltransferases will efficiently conjugate 2,4,5-TCP (Mebner et al. 2003). It is hypothesized that similar conjugation pathways occur for most halogenated phenols, including fluorinated and trifluoromethylated phenols; however, this pathway has only been demonstrated for acifluorfen, where intermediate 2-chloro-4-trifluoromethylphenol was glucosylated (Frear et al. 1983a).

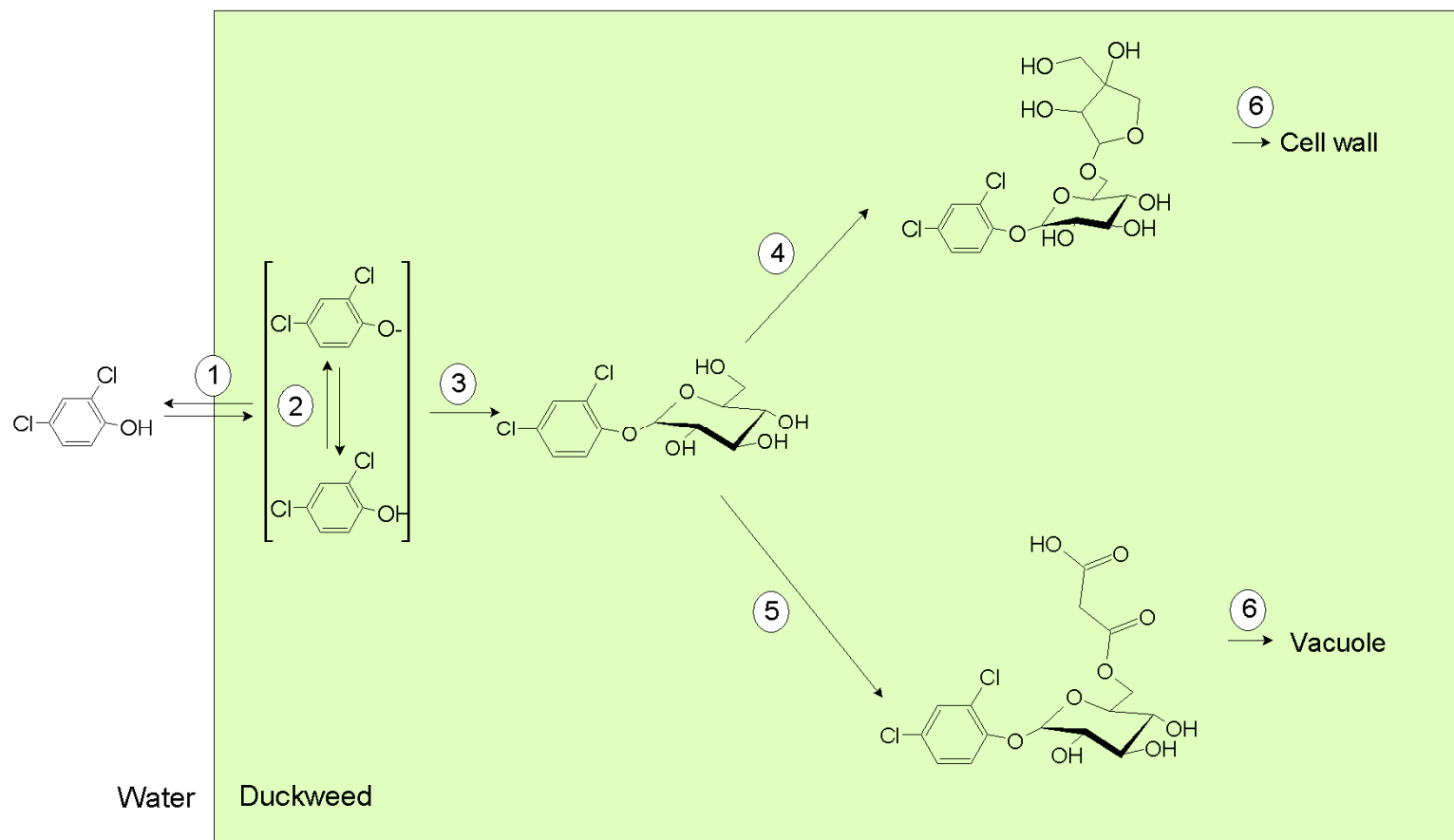


Figure 2.4. Depiction of uptake of 2,4-dichlorophenol by *L. minor*. Numbers represent processes associated with uptake: (1) abiotic partitioning; (2) deprotonation internal to the plant cytosol; (3) glycosylation of parent 2,4-DCP with glucose, or glycosylation, forming 2,4-dichlorophenyl-β-Dglucopyranoside; (4) glycosylation with apiose forming 2,4-dichlorophenyl-β-Dglucopyranosyl-(6→1)-β-D-apiofuranoside; (5) glycosylation with malonate forming 2,4-dichlorophenyl-β-D-(6-O-malonyl)glucopyranoside; and (7) sequestration into plant tissue. Figure derived from Day and Saunders (2004) and Tront and Saunders (2006).

Plants sequester phytometabolized products of organic pollutants in cell walls and vacuoles, in plant cells through Phase III metabolism. Occasionally, the nature of metabolites can indicate the final metabolite destination in plants. For example, 2,4-DCP is metabolized to 2,4-dichlorophenyl- β -Dglucopyranosyl-(6 \rightarrow 1)- β -D-apiofuranoside (DCPAG) (Day and Saunders 2004). Apiosyl conjugation strongly indicates that 2,4-DCPAG is further metabolized and then incorporated into cell walls, as apiose is uniquely associated with cell walls in *L. minor* (Roberts et al. 1967; Hart and Kindel 1970; Golovchenko et al. 2002). Similarly, conjugation of organic pollutants with malonyl residues, as was observed as a major metabolic pathway for detoxification of 2,4-DCP, suggests sequestration of pollutant metabolites in plant vacuoles (Komossa et al. 1995; Day and Saunders 2004).

Although specific transport proteins have not been identified, studies indicate that glucosylated compounds in plant cells are transported into vacuoles by either proton antiporter mechanisms (Martinoia et al. 2000) or directly energized ABC-like transporters (Klein et al. 1996). Studies with barley indicate that unique mechanisms exist for vacuolar uptake of flavonoid glucosides and herbicide glucosides (Klein et al. 1996). Vacuolar compartmentalization of herbicide glucosides, specifically hydroxyprimisulfuron-glucoside, was stimulated by addition of ATP to solution – indicating extensive energy requirements for compartmentalization of herbicide glucosides. However, vacuolar uptake of flavonoid glucosides was similar under nonenergized conditions and in presence of ATP, suggesting dissimilar vacuolar uptake mechanisms. Additionally, vacuolar uptake of flavonoid and herbicide glucosides differed in nucleotide specificities and response to ATPases inhibitors (Klein et al. 1996).

Examples of literature reports on plant metabolism of organic pollutants are provided in Table 2.3. A broad range of Phase I and II metabolites have been identified in multiple plant species. Phase III metabolism is typically indicated by presence of unidentified “bound” fractions, as detected by following radioactivity of radiolabeled

parent organic pollutants. The majority of reports prior to 1990 focused on agrochemicals and agronomically important plant species, including crops and weeds.

Plant metabolism of organic pollutants is frequently regulated by temperature. Removal of trinitrotoluene (TNT) by parrot feather and stonewort in batch studies indicated that formations of aminodinitrotoluenes and diaminonitrotoluenes increase with increasing temperatures up to 34 °C (Medina et al. 2000); however, as studies did not report results from axenic plant cultures or quantify microbial communities, increased degradation rates with increased plant density could have resulted from increased numbers of microorganisms. Plant metabolism of cyanide was also dependent on temperature, with greater temperature effects for weeping willow than Chinese elder; temperature coefficient values (Q_{10}), or the ratios by which removal rates changed with temperature increase of 10 °C, were 2.09 and 1.84, respectively (Yu et al. 2005).

Table 2.3. Examples of reports of phytometabolism of organic pollutants by terrestrial and aquatic plants. ¹Check indicates that cultures were described as sterile or axenic. ²Check indicates that studies utilized plant tissue cultures. Phase I, II, and III refer to transformation, conjugation, and sequestration phases (respectively) of plant metabolism, as described in detail in text.

Pollutant	Plant species	S/A ¹	TC ²	Phase			Metabolites	Reference
				I	II	III		
Phenols								
Phenol	<i>Lemna gibba</i>	✓			✓		Glucoside conjugation	(Barber et al. 1995)
Phenol	Maize, pea, pumpkin				✓		Peptide conjugation	(Chrikishvili et al. 1977; Ugrekhelidze et al. 1983)
Mono-, di-, and tri-atomic phenols	Broadbean				✓		Glucoside conjugation	(Pridham 1958)
2,4-DCP, 2,4,5-TCP	<i>Lemna minor</i>				✓	✓	Glucoside, apiose, and malonyl conjugation	(Day and Saunders 2004)
2,4-DCP	<i>Lemna minor</i> , <i>Glyceria maxima</i> , <i>Mentha aquatica</i> , <i>Myriophyllum spicatum</i> , <i>Hippuris vulgaris</i>				✓	✓	Glucoside, malonyl, acetyl, and unidentified pentosyl carbohydrate conjugation	(Pascal-Lorber et al. 2004)
2,4,5-TCP	<i>Lemna gibba</i>				✓		Glucoside conjugation	(Sharma et al. 1997)
2,4,6-TCP	Tomato					✓	Unidentified bound fraction	(Fragiadakis et al. 1981)
Pentachloro-phenol (PCP)	Soybean, spinach				✓		Glucoside conjugation	(Casterline Jr. et al. 1985)
PCP	Rice				✓		Glucoside conjugation	(Haque et al. 1978; Weiss et al. 1982)
PCP	Wheat, soybean				✓	✓	Glucoside and malonyl conjugation	(Schmitt et al. 1985a; Schmitt et al. 1985b)

Table 2.3 (continued)

Pollutant	Plant species	S/A ¹	TC ²	Phase			Metabolites	Reference
				I	II	III		
Explosives								
RDX, HMX	<i>Catharanthus roseus</i>	✓	✓			✓	Unidentified bound fraction	(Bhadra et al. 2001)
TNT	<i>Myriophyllum aquaticum</i>	✓	✓	✓			Oxidation products	(Bhadra et al. 1999)
TNT	<i>Myriophyllum aquaticum</i>			✓			Amine and azoxy products	(Wang et al. 2003)
TNT	Tobacco	✓	✓	✓	✓	✓	Reduction of nitro groups, glycoside conjugation, bound residues	(Vila et al. 2005)
2,4-dinitro-phenol	Maize, pea, pumpkin				✓		Peptide conjugation	(Arziani et al. 1983)
2-nitrophenol	Pea				✓		Peptide conjugation	(Ugrekheldidze and Arziani 1980)
Pesticides								
Acifluorofen	Soybean			✓	✓		Diphenylether bond cleavage; glutathione, cysteine, and glucose conjugation	(Frear et al. 1983a)
Alachlor	Maize				✓		Glutathione conjugation	(O'Connell et al. 1988)
Acetochlor	Maize, morning glory, field bindweed, cocklebur, soybean, mung bean, alfalfa				✓		Glutathione conjugation	(Breaux 1987)
Atrazine, chlorinated benzenes, DDT	Barley					✓	Unidentified bound fraction	(Trapp et al. 1990)

Table 2.3 (continued)

Pollutant	Plant species	S/A ¹	TC ²	Phase			Metabolites	Reference
				I	II	III		
Pesticides								
Atrazine	53 Gramineae species				✓		Glutathione conjugation	(Jensen et al. 1977)
Atrazine	Potato, wheat	✓	✓	✓	✓		Glutathione conjugation, <i>N</i> -deethylation	(Edwards and Owen 1989)
Atrazine	Resistant bluestem and switch grass, sensitive Indian grass and sideoats			✓	✓		Glutathione conjugation (resistant species) and <i>N</i> -deethylation (sensitive species)	(Weimer et al. 1988)
Atrazine	<i>Spartina alterniflora</i>			✓			Dealkylation, no hydroxy- derivatives	(Pillai et al. 1977)
6-Benzyl-aminopurine	Snapbean				✓		Riboside formation	(Ramina et al. 1979)
Buturon	Wheat			✓			Hydroxylation of methyl groups	(Haque et al. 1977)
Carbofuran	Barley			✓			Hydroxylation	(Penner and Early 1973)
Carbofuron	Strawberry			✓			Hydroxylation	(Archer et al. 1977)
Chlorimuron-ethyl	Soybean				✓		Homo-glutathione conjugation	(Brown and Neighbors 1987)
Chloroanilines	Wheat, soybean	✓	✓		✓	✓	Glucoside and malonyl conjugation	(Wilkner and Sandermann Jr. 1989)
Chlorotoluron	Wheat			✓			Hydroxylation, demethylation	(Gross et al. 1979; Cabanne et al. 1985)
DDT	<i>Elodea</i> , kudza						abiotic reduction by biochemically derived molecules	(Garrison et al. 2000)

Table 2.3 (continued)

Pollutant	Plant species	S/A ¹	TC ²	Phase			Metabolites	Reference
				I	II	III		
Pesticides								
Diclofop, diclofop-methyl	Wheat, oat seedlings			✓	✓		Hydroxylation and glucoside conjugation	(Jacobson and Shimabukuro 1984; Shimabukuro et al. 1987; McFadden et al. 1989; Tanaka et al. 1990)
2,4-Dichloro-phenoxyacetic acid (2,4-D)	<i>Glycine</i> species			✓	✓		Hydroxylation, glucoside conjugation, amino acid conjugation	(White et al. 1990)
2,4-D	Maize, tobacco, carrot, sunflower	✓	✓		✓		Glutamic and aspartic acid conjugation	(Feung et al. 1975)
2,4-D	Rice	✓	✓		✓		Glucosyl ester formation	(Feung et al. 1975)
2,4-D	Soybean	✓	✓		✓		Glutamic and aspartic acid conjugation	(Davidonis et al. 1978)
2,4-D	Wild buckwheat, leafy spurge, yellow foxtail			✓			Hydroxylation	(Fleeker and Steen 1971)
2,4-D	Wheat				✓		Glucosyl ester conjugation	(Chkanikov et al. 1976)
Diphenamid	Pepper seedlings			✓			Hydroxylation	(Hodgson and Hoffer 1977)
Diphenamid	Tobacco	✓	✓	✓			Hydroxylation	(Burrows and Leworthy 1976)
Diuron	Sugar cane			✓	✓		Hydroxylation and glucose conjugation	(Liu et al. 1978)
Ethephon	<i>Hevea brasiliensis</i>				✓		Glucoside conjugation	(Audley 1979)

Table 2.3 (continued)

Pollutant	Plant species	S/A ¹	TC ²	Phase			Metabolites	Reference
				I	II	III		
Pesticides								
Etirimol	Barley			✓	✓		Hydroxylation and glycoside conjugation	(Harborne 1977)
Geraniol	Apple				✓		Glucoside conjugation	(Wills and Scriven 1979)
Metribuzin	Soybean				✓		Glucoside and homo-glutathione conjugation	(Frear et al. 1985)
Metribuzin	Tomato				✓	✓	Glycoside and malonyl conjugation	(Frear et al. 1983b; Smith et al. 1989)
Monolinuron	Spinach			✓			Hydroxylation	(Schuphan and Ebing 1978)
Monuron	Gossypium cotton			✓	✓		Hydroxylation and glucoside conjugation	(Frear and Swanson 1972)
Phenoxyacetic acid	Oat	✓	✓	✓			Hydroxylation	(Hutber et al. 1978)
Phenoxyacetic acid	Maize and snapbean				✓		Conjugation with low molecular weight peptides	(Kakhniashvili et al. 1979)
Phenoxyacetic acid	Soybean	✓	✓	✓	✓		Hydroxylation, glucoside and glycosidic ester conjugation	(Laurent and Scalla 1999)
Preilachlor	Rice				✓		Glutathione conjugation	(Han and Hatzios 1991)
Propachlor	Soybean				✓		Homo-glutathione conjugation	(Lamoureux and Rusness 1989)
Saligenin	Broad bean				✓		Glucoside conjugation	(Pridham 1958)

Table 2.3 (continued)

Pollutant	Plant species	S/A ¹	TC ²	Phase			Metabolites	Reference
				I	II	III		
Pesticides								
Saligenin	<i>Datura innoxia</i>	✓	✓		✓		Glucoside conjugation	(Tabata et al. 1976)
Simazine	Poplar, walnut			✓			Dealkylation; no hydroxy derivatives	(Wichman and Byrnes 1975)
Terbacil	Alfalfa			✓			Hydroxylation	(Rhodes 1977)
Terbutiuron	Sugar cane			✓			Hydroxylation	(Loh et al. 1978)
Other organic pollutants								
Alkanes				✓			Oxidation to CO ₂	(Durmishidze and Ugrekhelidze 1968)
Benzene, toluene	Maize, peas, pumpkins	✓		✓			Cleavage to aliphatic products, hydroxylation	(Ugrekhelidze et al. 1997)
Benzo[a]pyrene	Parsley, soybean	✓	✓	✓	✓		Oxidation to aliphatics, glutathione conjugation	(Trenck and Sandermann 1978)
Ethanol	Mung bean				✓		Glucoside conjugation	(Middleton et al. 1978)
Ethylene dibromide	<i>Leuceana leucocephala</i>			✓			Degradation and bromide release	(Doty et al. 2003)
Hydroquinone	Wheat				✓		Glucoside conjugation	(Harborne 1977)
Perchlorate	Poplar			✓			Reduction	(Van Aken and Schnoor 2002)
Polycyclic aromatic hydrocarbons				✓			Hydroxylation	(Devdariani 1988)
Trichloro-ethylene	Poplar	✓	✓			✓	Unidentified bound fraction	(Shang and Gordon 2002)
Trichloro-ethylene	Tropical tree (<i>Leuceana leucocephala</i>)			✓			Hydroxylation forming trichloroethanol	(Doty et al. 2003)

Fate of plant metabolites of organic pollutants

Sequestration of organic pollutants by aquatic plants prompts concerns about bioavailability and ultimate fate of sequestered pollutants. Case studies have indicated that conjugated and “bound” pollutants are available to herbivores; the estimated bioavailability value of many pesticides sequestered by crop plants exceeds existing maximum residue levels for food crops (Sandermann 2004a). For example, approximately 70% chlorpyrifos-methyl was incorporated into bound residues in wheat; up to 80.5% of bound chlorpyrifos methyl was subsequently bioavailable to mammals via consumption of wheat. Bioavailability percentages for pesticides that have been sequestered by crop plants ranged from 1.6% to 81.7% (Sandermann 2004a), indicating that exposure of sequestered pollutants through consumption of crops can range from negligible to substantial based on many factors, including plant species, pesticide, and pesticide application rate.

Sequestration of organic pollutants by aquatic plants in wetlands also has implications for ultimate fate of pollutants and exposure of humans to sequestered pollutants. Turn-over of plants and incorporation of plant detritus into wetland sediments suggests that sequestered pollutants will be incorporated into anaerobic wetland sediments. Bioassays utilizing *Desulfitobacterium* species strain Viet 1 in anaerobic microcosms studies have demonstrated that 2,4-DCP sequestered by *L. minor* is readily available upon plant death. *Desulfitobacterium* sp. strain Viet 1 selectively dechlorinates 2,4-DCP to 4-chlorophenol (4-CP), which was utilized as an indicator of availability of 2,4-DCP sequestered by *L. minor* to microbial degradation. Approximately 26% of sequestered 2,4-DCP was released in autoclaved controls, while $43.5 \pm 1.4\%$ of sequestered 2,4-DCP sequestered by *L. minor* was reduced to 4-chlorophenol by *Desulfitobacterium* sp. strain Viet 1 in bioassays (Tront et al. 2006). Consequently, approximately 17% of sequestered 2,4-DCP was released upon microbial decomposition

of plant tissues. However, over 50% of sequestered 2,4-DCP was not released during the 120 day experiment. Consequently, a significant portion of sequestered pollutants may remain bound in wetland sediments for long periods of time.

Research on fate of organic pollutants in plant systems has focused on agronomically important plants, especially terrestrial crops and weeds; research on uptake and metabolism of organic pollutants with regards to aquatic plants is more limited. However, uptake and metabolism of organic pollutants by aquatic plants may differ substantially due to differences in physiologies, partitioning processes within and into plants, and metabolism. For example, studies on uptake of halogenated phenols by *L. minor* indicates that relationships for uptake of organic pollutants by terrestrial plants are not applicable to uptake by aquatic plants (Tront et al. 2007). Additionally, as shown in Table 2.3, the aquatic plant *Myriophyllum aquaticum* metabolized TNT via Phase I transformations, including oxidation and reduction of nitro- groups (Bhadra et al. 1999; Wang et al. 2003), while tobaccos plants metabolized TNT via Phase I, Phase II, and Phase III reactions, resulting in reduction of nitro-groups, glycoside conjugates, and ultimately bound residues (Vila et al. 2005). Therefore, studies examining interactions between organic pollutants and aquatic plants are crucial to understanding fate of organic pollutants in wetlands. Use of plant tissue cultures is one avenue through which increased understanding of phytometabolism has been achieved in terrestrial systems (e.g., 14 studies summarized in Table 2.3 utilized plant tissue cultures). The following section discusses basics of plant tissue culture, plant tissue culture research in Lemnaceae, and potential applications for plant tissue culture in increasing understanding of interactions between organic pollutants and Lemnaceae.

Plant tissue culture

History of plant tissue culture

Principles of plant tissue culture were first recognized in the early 19th century when Schleiden (1838) and Schwann (1839) theorized that plant cells are capable of autonomous and totipotent growth. Totipotency describes the ability of an individual cell to form all of the cell types in the adult organism, while autonomous indicates the ability of individual cells to develop independently from the whole organism. However, initial attempts at plant tissue culture were largely unsuccessful for the next 130 years (Gautheret 1983). The first sustainable plant tissue cultures, root cultures of tomatoes, were initiated in 1934 (White 1934). More than a decade later, Ball fathered micropropagation of plants by demonstrating which parts of shoot meristems were able to produce entire plants (Ball 1946). Meanwhile, the importance of auxins, including indoleacetic acid, for unlimited culture of plant tissues was simultaneously demonstrated in the United States and France by White (1939), Nobécourt (1939), and Gautheret (1939). Caplin and Steward (1948) subsequently discovered the importance of cytokinins to tissue proliferation through observations of active cellular proliferation in carrot explants when coconut milk was added to culture medium. The active ingredient in coconut milk, identified as ribosylzeatin (Letham 1974), belongs to a class of plant hormones called cytokinins. Auxin:cytokinin ratios are crucial determinants of morphology and differentiation in plant tissue cultures. High auxin:cytokinin ratios typically promote disorganized growth or root formation, while low ratios typically promote shoot development (Warren 1992). Gibberelins, a third class of plant hormones, were subsequently discovered to increase vascular differentiation in plant tissue cultures (Gautheret 1966).

Basics of plant tissue culture

Through various combinations of phytohormones and/or plant hormones, multiple forms of plant tissues can be cultured from parent plants. Forms of plant tissue culture are characterized by the level of organization of plant cells. More organized systems of plant tissue culture include root cultures, micropropagation, and anther and pollen cultures. Less organized systems of plant tissue culture include callus cultures, cell suspensions, somatic embryo cultures, and protoplast cultures. A callus is an amorphous mass of actively dividing, thin-walled parenchyma cells. Though typically initiated and maintained on medium containing nutrients, carbohydrates, vitamins and phytohormones and/or plant hormones, infection of plant tissue with *Agrobacterium tumefaciens* can also promote callus growth (Gautheret 1983). Cell suspension cultures are initiated through suspension and constant shaking of callus cultures in liquid medium. Ability to initiate cell suspension cultures depends on the nature of the callus inoculum; cell suspension cultures are more easily obtained with small and friable callus than with hard callus (Warren 1992).

An outline of common procedures for development of plant tissue cultures is provided (Figure 2.4). While much of the following discussion is applicable to many types of plant tissue culture, discussion will focus on indirect morphogenesis to initiate callus cultures and subsequent suspension of callus in liquid medium to culture cell suspensions. Plant tissue culture first requires establishment of aseptic plants or plant tissues for use as explants. Disinfection of plants and plant tissues typically utilize either bleach or hydrogen peroxide as disinfecting agents (Pullman 2004). Two major cell types are typically used as explants to initiate culture of plant cells: meristematic and mesophyll cells (Warren 1992). Meristematic cells are characterized by thin walls, small (ca. 20 μm) and similar sizes, high metabolic activity, ability to sustainably divide, and high morphogenic potential. Mesophyll, or chloroplast-containing cells, are typically larger (approximately 50 μm in length) and are commonly associated with leaf

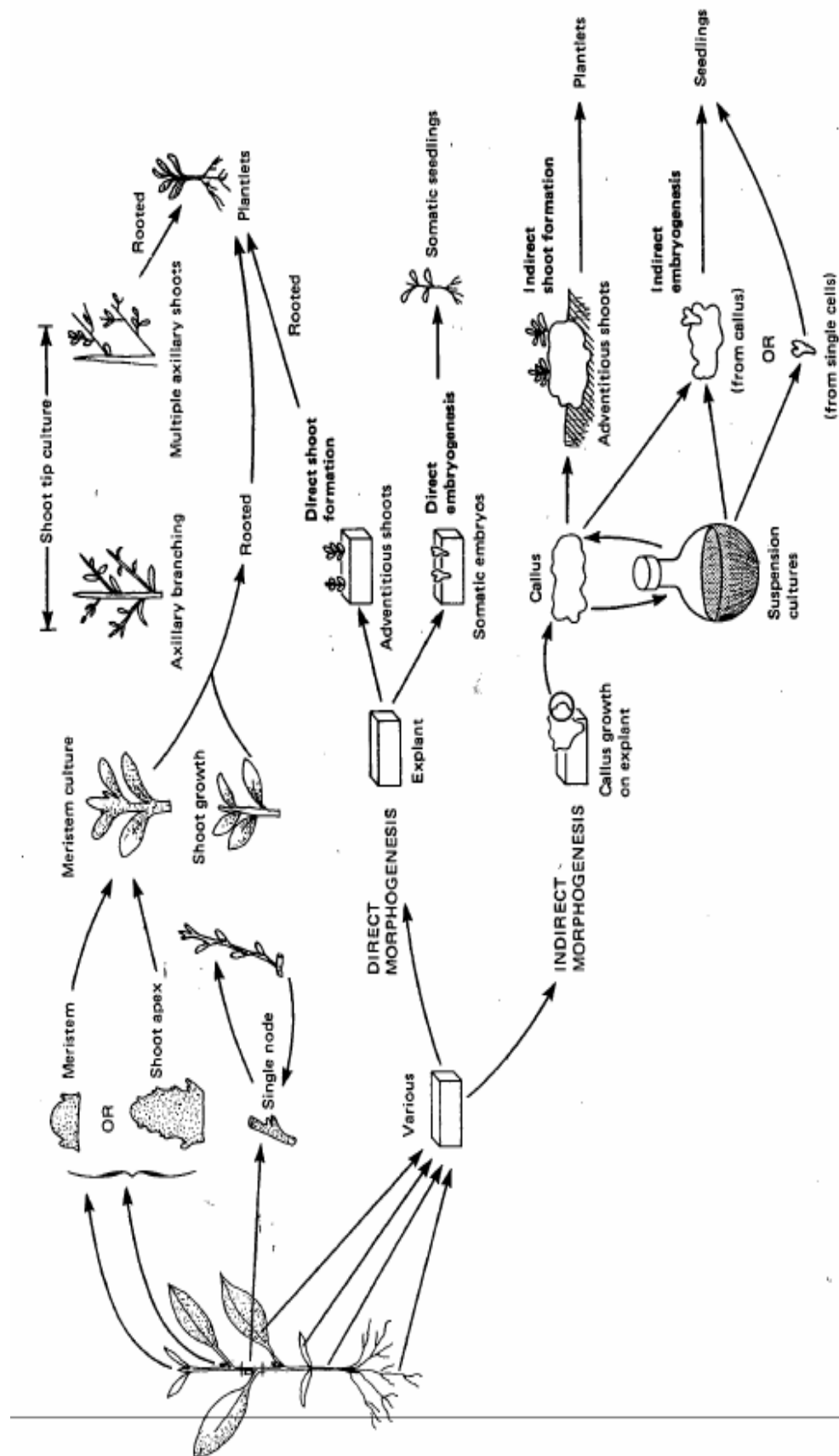


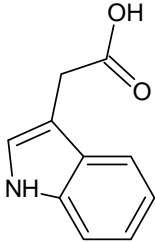
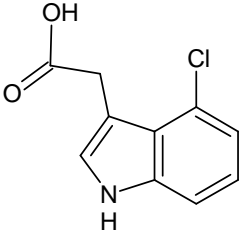
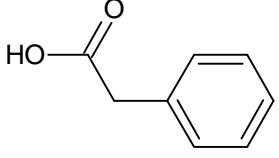
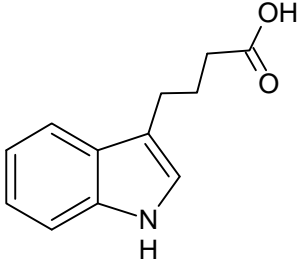
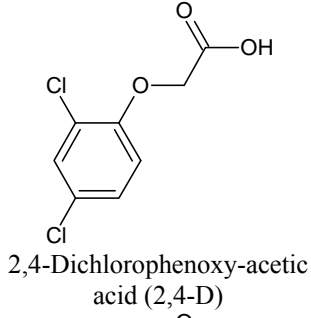
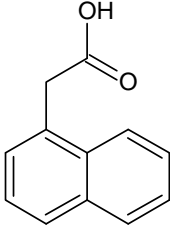
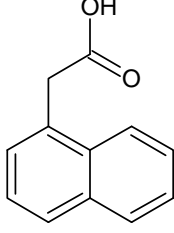
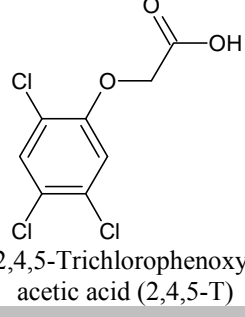
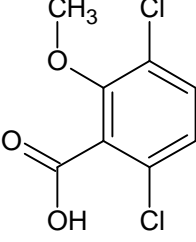
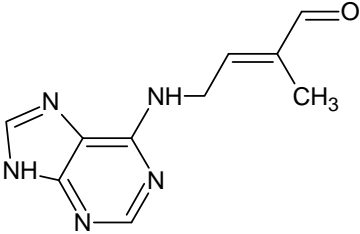
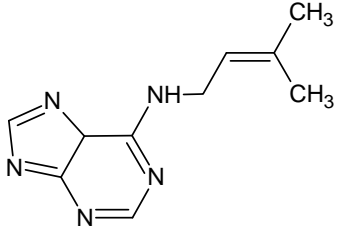
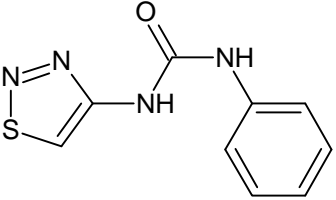
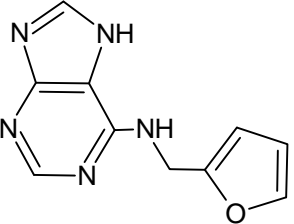
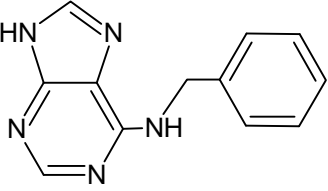
Figure 2.4. Depiction of common plant tissue culture methods. Taken from Pullman (2004).

tissues. Successful initiation of callus or cell culture is highly dependent on explant genotype, origin, and health (Murashige 1974; Warren 1992).

Plant tissue medium typically consists of inorganic salts, organic substances, and (frequently, but not always) natural complexes (Murashige 1974). Multiple formulations for basal inorganic salt media have been developed, including MS (Murashige and Skoog 1962), NN (Nitsch and Nitsch 1969), SH (Schenk and Hidebrandt 1972), and B5 (Gamborg et al. 1976) medium. Organic substances in plant tissue medium include carbohydrates, vitamins, amino acids and amides, nitrogen base (i.e., adenine), and growth regulators (Murashige 1974). Two to three percent sucrose is typically added as a carbohydrate source, while thiamin, inositol, nicotinic acid, and pyridoxin are added as vitamins to promote growth. Amino acids that are commonly added to plant tissue medium include arginine, aspartic acid/asparagine, glutamic acid/glutamine, and tyrosine. Natural complexes, including yeast extracts, hydrolyzed protein preparations, fruit juices, and coconut milk, are occasionally added if defined medium preparations do not adequately support plant tissue growth. The pH of plant tissue medium is typically adjusted to pH 5.0 – 6.0 (Murashige 1974).

Successful plant tissue culture is largely dependent on appropriate selection of natural plant hormones or synthetic phytohormones for each specific application. Names and structures of commonly used auxins and cytokinins are provided in Table 2.4. Quantification of hormone or phytohormone concentrations inside culture cells is complicated by complex relationships between phytohormones or hormones and culture cells, including (i) cellular metabolism and storage of exogenous hormones or phytohormones, (ii) changes in synthesis of endogenous hormones due to medium substrates, (iii) carry-over of hormones or phytohormones from previous treatments, and (iv) microenvironments in clusters of culture cells (Warren 1992). Habituation, or the ability of cultured cells to synthesize endogenous hormones and consequently decrease effects of exogenous hormones or phytohormones, frequently increases with increasing

Table 2.4. Common hormones and phyto regulators used in plant tissue cultures

Native auxins		
		
Indole-3-acetic acid (IAA)	4-chloroindole-3-acetic acid (4-Cl-IAA)	2-phenylacetic acid (PAA)
Synthetic auxins		
		
Indole-3-butyric acid (IBA)	2,4-Dichlorophenoxy-acetic acid (2,4-D)	α-Naphthalene acetic acid (α-NAA)
		
Picloram	2,4,5-Trichlorophenoxy-acetic acid (2,4,5-T)	Dicamba
Native cytokinins		
		
Zeatin	N ⁶ -(3-methylbut-2-enyl)adenine (2-iP)	
Synthetic cytokinins		
		
Thiadiazuron (TDZ)	Kinetin	6-benzyladenine (BA)

time in culture (Warren 1992). Habituation can thus limit sustainable culture of callus and cell suspension cultures.

Variability with respect to structural, metabolic, and genetic features is a crucial characteristic of plant cells in culture and results from an absence of control systems that operate in the whole plant (Warren 1992). Multiple classes of plant cells are observed in culture, including small meristematic cells and large, vacuolated cells (Warren 1992). Growth of plant cells and callus is typically considered either “unorganized” or “dedifferentiated”; however, differentiation and organization are present in plant cell and callus culture. Plant cells in culture still possess subcellular organelles, including cell walls, plasma membranes, vacuoles, and plastids (Warren 1992). Additionally, tissue culture cells, especially callus cells, are characterized by numerous or large vacuoles, thin cytoplasm, large and diverse cell shapes, and only barely visible nuclei (Murashige 1974).

Plant tissue cultures are typically heterotrophic; however, photoautotrophic cultures can be obtained in the presence of high concentrations of carbon dioxide (~1%) and high light intensity (10,000 lux). Addition of carbohydrates and 2,4-dichlorophenoxyacetic acid to culture medium inhibits chlorophyll synthesis and subsequent photosynthetic oxygen production (Warren 1992). Additionally, callus cultures grown with carbon sources and light exposures often consist of a combination of heterotrophic and phototrophic properties (e.g., both white and chlorophyll-containing green callus may be present).

Cell suspension cultures are inherently genetically heterogeneous and instable; genotypic variability may decrease with culture time, but the resulting genotypes are likely divergent from the parent genotype of the original explant (Warren 1992). Somaclonal variation, a form of *in vitro* mutagenesis, is one source of genotype divergence in callus and cell suspension cultures (Loh 1992; Warren 1992). Somaclonal variation typically increases with length of time in culture.

Development of effective procedures for plant tissue culture ushered in numerous applications for plant tissue cultures. Micropropagation of roots and shoots has greatly advanced agriculture; cultivation of meristematic root or shoot tissues of infected plants yields healthy, uninfected plants (Gautheret 1983). Additionally, micropropagation allows for clonal propagation of important agriculture and horticultural plants. Selection of beneficial phenotypes, via externally-induced and intrinsic mutagenesis in callus and cell suspension cultures, increased commercialization of plants resulting from plant tissue cultures. Selection of phenotypes with increased herbicide tolerance, salt tolerance, heavy metal tolerance, disease resistance, and amino acid overproduction has advanced crop development; however, success has been mixed and many reports of regeneration of mutants have not been supported by field tests (Loh 1992). Production of secondary metabolites in metabolically-engineered plant tissue cultures, predominantly in dedifferentiated cell cultures, is another arena of active research. Plant cell suspension cultures have been used in conjunction with metabolic engineering to manipulate plant cell production of flavonoids to modify flower colors, down-regulate lignin synthesis to improve cellulose content for paper production, and secondary production of pharmaceuticals (Bourgaud et al. 2001).

Detection of microbial contaminants in plant tissue culture typically relies on visual observation and inoculation of microbial growth medium with small portions of plant tissue. Epiphytic and endophytic microorganisms are a major source of microbial contamination in plant tissue cultures (Reed and Transprasert 1995). Molecular tools, including polymerase chain reaction–restriction fragment length polymorphism, have also been used to detect bacterial contaminants in symptomless plant tissue cultures (Isenegger et al. 2003). Reduced visibility, due to opaque gelling agents in solidified medium, complicates visual detection of bacterial contaminants (Debergh and Vanderschaeghe 1988); clear and color-less gelling agents, such as Phytigel (Sigma-Aldrich, St. Louis, MO), can simplify visual detection of microbial contaminants on

solidified plant tissue medium.

Plant tissue culture of Lemnaceae

While initial research on plant tissue culture focused on agriculturally and horticulturally important plants, research into development of plant callus cultures of Lemnaceae species began in the 1970s. The majority of research into plant tissue culture of Lemnaceae has focused on callus induction and plant regeneration for the purpose of physiological studies and genetic transformations. Reports of cell suspension cultures of Lemnaceae were not found after extensive literature review. As research presented in this thesis only involves callus induction and growth, the following literature review focuses on callus induction and growth and does not review established procedures for regeneration of plants from callus or genetic transformation of callus-derived cultures.

Callus was first induced from Lemnaceae in *L. gibba* L. using MS medium with 10 mg/L 2,4-D and 1 mg/L 2-iP (Chang and Chiu 1976). Callus was first observed on 50% of explants after 3 weeks, with formation of large, irregularly shaped callus at the end of eight weeks. Two types of callus were observed: yellow, irregularly shaped, friable, and fast growing callus and green, compact, and slow growing callus. Spontaneous formation of differentiated structures resembling roots or embryos were observed (Chang and Chiu 1976).

Extensive studies on optimizing callus induction, growth, and frond regeneration in *L. gibba* explored the effects of basal medium, plant growth regulator type and concentration, sucrose, and light (Moon and Stomp 1997). Of 2,4-D, NAA, IAA, and IBA, 2,4-D was most efficient at inducing callus, especially at high auxin concentration (i.e., 20 or 50 μ M vs. 2 μ M). Sucrose and light were required for callus induction and callus growth. Callus induction frequency was greater on MS (Murashige and Skoog 1962) medium than on NN (Nitsch and Nitsch 1969), SH (Schenk and Hidebrandt 1972), and B5 (Gamborg et al. 1976) medium. Callus proliferation was enhanced by addition of

10 μ M NAA, 10 μ M gibberellic acid 3 (GA₃), and 2 μ M BA to medium containing 1 or 20 μ M 2,4-D. However, even on optimized medium, callus induction frequency was less than 10% and growth was described as slow (Moon and Stomp 1997). Similar types of *L. gibba* callus were observed by Moon and Stomp (1997) as were observed by Chang and Chiu (1976) – relatively dedifferentiated, pale green to greenish yellow, slow growing callus and dark green, fast growing callus.

Callus induction and plant regeneration in *L. gibba* var. *Hurfeish* was further investigated by Li et al. (2004). Callus induction and growth was enhanced by pretreatment of fronds on B5 medium containing 1.5% galactose, 50 mg/L dicamba, and 2 mg/L BA for two to four weeks prior to transfer to callus induction medium containing B5 basal nutrients, 1% sucrose, 50 mg/L dicamba, and 2 mg/L BA. Pretreatment increased callus induction from 10% to approximately 75%. Subsequent callus growth required transfer to B5 medium with 1% sucrose, 10 mg/L *para*-chlorophenoxyacetic acid, 2 mg/L picloram, and 2 mg/L 2-iP. Results for *L. gibba* var. *Hurfeish* indicate that successful callus induction for Lemnaceae species may require unique medium for explant pretreatment, callus induction, and callus growth (Li et al. 2004), and were in stark contrast to previous callus induction studies (Chang and Chiu 1976).

Plant tissue culture of Lemnaceae has also focused on callus induction and plant regeneration in *L. minor* L. Initial studies utilized *L. minor* L. fronds that have been grown under laboratory conditions since for more than 20 years (Frick 1991). Pretreatment on medium containing 2 mg/L 2,4-D and 0.2 mg/L 2-iP was required prior to callogenesis on medium containing 5 mg/L 2,4-D and 0.5 mg/L 2-iP. Carbohydrate source was crucial in callus growth, with sucrose, galactose, melibiose, or mannitol supporting increased callus growth and, in the case of galactose, in inhibiting frond growth (Frick 1991). Callus induction in *L. minor* L. was also achieved on MS medium with 45 μ M 2,4-D when fronds were surgically injured in the meristematic region (Stefaniak et al. 2002). Surgical injury was required for callus induction; callus was

induced from 89.1% surgically induced fronds, but was not induced from whole plants, frond halves, or basal root segments. As compared with previous studies, Stefaniak et al. (2002) induced callus only in the dark and did not compare induction frequency or callus growth in light and dark conditions. Callus was described as cream-yellow, soft, and friable with sporadic growth of white, hard, and compact callus. *L. minor* L. callus was also cultured on MS medium with 1.0 mg/L 2,4-D and 0.5 mg/L BA under dark conditions for the purposes of polysaccharide biochemistry studies; however, a description of callus was not provided (Gunter et al. 2004).

While the majority of callus induction research has focused on *Lemna* genus of Lemnaceae, Li et al. (2004) describes callus induction protocols for *L. punctata* 8717 (previously known as *Spirodela punctata*) and *Spirodela oligorrhiza* SP. Developed protocols for callus induction utilized different basal nutrient medium, carbohydrates, auxins, and cytokinins, indicating that callus induction in may be highly genus specific. As with previous studies of callus induction in *Lemna* species, the research goal was to develop protocols for callus induction and regeneration of plants from induced callus; consequently, callus was described as soft and likely unsuitable for cell suspension culture (personal correspondence).

Studies on necessity of light for Lemnaceae callus induction and growth have conflicting results. Multiple studies utilized dark conditions for induction and growth of callus (Stefaniak et al. 2002; Gunter et al. 2004), while additional studies report slower growth of callus under dark conditions as compared to growth under either 24:0 or 16:8 light:dark photoperiod (Chang and Chiu 1976; Frick 1991). However, researchers also report negligible growth or callus senescence under dark conditions (Moon and Stomp 1997). Moon and Stomp (1997) proposed that incidental light introduction to “dark” conditions may account for the discrepancy between their and other results. Origin and genotype of explant fronds may provide another explanation for the discrepancy in capabilities of researchers to induce and grow callus in the dark, as reported callus

induction frequencies with light exposure were substantially lower for Moon and Stomp (1997) than for other researchers (Chang and Chiu 1976; Stefaniak et al. 2002; Li et al. 2004).

Plant tissue cultures to study pollutant metabolism and phytotoxicity

Aquatic plant studies of environmental fate and toxicity can be technically challenging because of complex interactions between aquatic plants and the environment. For example, degradation by microorganisms on plant surfaces can attenuate pollutants and interfere with assessments of plant uptake. However, disinfection techniques to remove surface microorganisms can decrease plant activity and disrupt symbiotic microorganism-plant relationships, yielding uptake rates that are lower than for healthy plants. Additionally, while whole-plant cultures are beneficial in assessing the overall process of contaminant removal in aquatic plant systems, sorption and mass-transfer limitations in whole-plant studies hinder detailed analysis of internal metabolic reaction rates.

Plant tissue culture has partially addressed many of these issues. Cell suspension cultures are well-suited to study metabolic degradation and conjugation of pollutants (Sandermann et al. 1984; Harms 1992; Babczinski 1999; Schaffner et al. 2002). Because of high surface area to volume ratio in plant cell suspension cultures, partitioning into cells is rapid and intrinsic process velocities, half-rate coefficients, and enzyme inhibition levels for varying contaminant concentrations should be easier to quantify. Theoretically, by growing cell suspension cultures under various conditions, metabolites can be associated with distinct plant processes. For example, comparing cell cultures grown heterotrophically (with sucrose) with cultures grown autotrophically (in the light) can identify photosynthetic metabolites. Protoplasts created from cell suspension cultures theoretically eliminate cell wall incorporation as a metabolic process. Finally, analysis of growth in plant cell cultures is simple and utilizes dry-mass measurements,

spectrophotometric readings, and vital-stain procedures (Dixon and Gonzales 1994; Dodds and Roberts 1995). Use of plant tissue cultures may also facilitate modeling of phytometabolism of organic pollutants. Suspended spherical cell aggregates of *Populus deltoids* x *P. nigra* (DN34) were used to assess three models of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) phytometabolism (Mezzari et al. 2004). Phytometabolism studies utilizing plant tissue cultures have been indicated previously (Table 2.3); studies that address the applicability and validity of plant tissue cultures studies in plant metabolism research are discussed further here.

Early studies on use of plant tissue cultures explored metabolism of 2,4-D, 2,4,5-T, hexachlorobenzene (HCB), pentachlorophenol (PCP), diethylhexylphthalate, benzo[α]pyrene, and DDT in *Glycine max* L. (soybean) and *Triticum aestivum* L. (wheat) cell suspension cultures (Sandermann et al. 1984). Cell suspension cultures had been established for greater than ten years prior to experimentation. Similar metabolites were found in cell cultures as had been previously reported in literature; however, relative proportions of metabolite classes differed and metabolism was enhanced in cell cultures. Results were reproducible with standard deviations of ± 10 to 20% (Sandermann et al. 1984).

Studies utilizing carrot, barley, and tomato cell suspension cultures indicated qualitatively similar species-specific toxicities in whole plants and in cell suspension cultures (Harms 1992). Additionally, uptake and metabolism of 4-chloroaniline, 4-nonylphenol, phenanthrene, 2,2',5,5'-tetrachlorobiphenyl, phenanthrene, benz[α]pyrene, and dibenz[α,η]anthracene was qualitatively similar in whole plants and cell suspension cultures (Harms 1992; Harms 1996). Length of plant tissue culture prior to experimentation was not explicitly stated, but was implied to be greater than five years. Furthermore, metabolic products were defined as polar, non-polar, or bound and detected using carbon isotope labeling – advanced analytical instruments were not used to more

fully identify metabolites.

Metabolism of the pesticide 4-amino-5-methyl-2-(*tert*-butylaminocarbonyl)-1,2,4-triazolin-3-one by *N*-glycosidation was observed in *Beta vulgaris conditiva* Alef (red beet) and *Glycine max* Merr cv *Merille* v *Mandarin* (soybean) cell suspension cultures (Babczinski 1999). The pesticide was completely metabolized in red beet cell suspension cultures, forming parent-*N*-glucosides, parent-*N*-glucoside-2'-*O*-sulfate, parent-*N*-glucoside-2'-*O*-glucuronide, and other products; pesticide metabolism was substantially less in soybean cell suspension cultures, with 77.4% of pesticide remaining in parent form. As red beet is tolerant to this pesticide, while soybean is not, increased metabolism in red beet versus soybean cell suspension cultures was considered an indication that plant cell cultures could be used as models for biochemistry of crop selectivity (Babczinski 1999). However, Babczinski (1999) notes that "very rare" plant metabolites, including a 2'-*O*-sulfated glycoside and a 2'-*O*-linked glucuronic acid containing disaccharide, were observed in red beet cell suspension cultures; as cell suspension studies were not conducted in parallel with whole plant studies, verification of the "very rare" plant metabolites in whole plant systems was not possible.

Plant cell suspension cultures have also been used in conjunction with advanced analytical instruments to detect organic pollutants and plant metabolites *in vivo*. Ten metabolites of the fluorinated fungicide *N*-ethyl-*N*-methyl-4-(trifluoromethyl)-2-(3,4-dimethoxyphenyl)benzamide were detected using ¹⁹F NMR on extracts of *Acer pseudoplatanus* L. cell suspension cultures; five of which were subsequently identified with thin layer chromatography and mass spectra analysis (Serre et al. 1997). Phytometabolism of *N*-ethyl-*N*-methyl-4-(trifluoromethyl)-2-(3,4-dimethoxyphenyl)-benzamide primarily proceeded through ring demethoxylation and N-dealkylation.

Plant cell suspension cultures have also been used to assess phytotoxicity of organic pollutants. Toxicities of detergent and diclofop to soybean (*Glycine max* (L.) Merr. 'Wilkin') and einkorn (*Triticum monococcum* L.) were assessed in cell suspension

cultures (Davis et al. 1984). Multiple endpoints, including settled cell volumes, packed cell values, absorbance at 525 nM of sonicated aliquots, dry weights, and conductivity and pH of culture medium. Settled cell volumes, conductivity, and dry-weight changes were most indicative of phytotoxicity. Phytotoxic effects of 4-chloroaniline, 3,4-dichloroaniline, phenanthrene, and 4-nonylphenol were measured in carrot, barley, and tomato cell suspension cultures; as responses in each cell suspension culture (i.e., carrot, barley, tomato) exhibited similar toxicities as corresponding axenic plant cultures, Harms (1992) concluded that plant cell suspension cultures were good systems for assessing phytotoxicity.

Extensive literature review did not find reports of using callus, in unsuspended form, to identify or quantify metabolism or phytotoxicity of organic pollutants. However, growth of Loblolly pine embryo growth on solidified medium containing variety of organic acids was quantified to assess the benefits of organic acid addition to embryonic cell growth (Pullman 2004; Pullman et al. 2006). Embryos were plated on 24-well plates, with a minimum of 48 replicates for statistical validity, and diameter of growth was measured after five to seven weeks. Furthermore, studies on use of plant tissue cultures from aquatic plants to study phytometabolism of non-agricultural pollutants in water sources were also not found. Consequently, thesis research explores use of Lemnaceae callus cultures for assessing interactions between fluorinated organic pollutants and Lemnaceae, including phytotoxicities and phytometabolism of fluorinated organic pollutants by Lemnaceae plants and callus cultures.

Descriptions of organic pollutants

While many organic pollutants, such as pesticides, are known ecosystem and human health hazards, the lack of knowledge of the hazards and environmental fates of most organic pollutants is overwhelming. As of September 2003, Chemical Abstract Services had indexed over 22 million organic and inorganic chemicals. Six million of

these were commercially available; however, only 227,000 of these chemicals were inventoried or regulated by any agency or government worldwide. In other words, only 4% of commercially available chemicals, and only 1% of known chemicals, are monitored (Daughton 2003).

In addition to use and consumption, humans are exposed to organic pollutants through a variety of pathways. Through consumption of meat, fish, fruits, vegetables, and drinking waters, humans are potentially exposed to organic pollutants that bioaccumulate in food webs or that persist through natural and engineered water treatment processes. Pharmaceuticals and personal care products are directly and indirectly (through human excretion) introduced to water systems via sewage collection and treatment. During wastewater treatment, organic pollutants may be transformed or degraded, frequently yielding other organic pollutants of concern. For example, triclosan photodegrades to form chlorinated phenols, dioxins, and furans (Sanchez-Prado et al. 2006). Pesticides and other organic pollutants may also enter water systems via stormwater or agricultural run off.

Attempts to model the accumulation of organic pollutants, through vegetation, cattle, and fish, indicate that organic pollutants with octanol-water partitioning coefficients of 10^2 to 10^{11} and octanol-air partitioning coefficients of 10^6 to 10^{12} exhibit high environmental bioaccumulation potential, as defined by the quantity of pollutant in humans divided by the quantity of pollutant in the environment (Czub and McLachlan 2004). Organic pollutants with these properties include 2,4-D, hexachlorobenzene, and atrazine.

Worldwide and national water shortages are expected to increase substantially during the next few decades; the U.S. EPA estimates that 36 states will suffer water shortages by 2013. Increased demand for water is increasing demand for reclamation of wastewaters and increasing potential exposures of humans to organic pollutants through drinking and irrigation waters. For example, concentrations of pharmaceuticals in soils

increased when reclaimed wastewaters were used for irrigation of city and golf course lands; results indicated that many pharmaceuticals in reclaimed wastewaters may persist for months in irrigated soils (Kinney et al. 2006).

The focus of the following literature review on experimental organic pollutants is to:

1. Note prevalence of experimental organic pollutants in water systems
2. Outline potential hazards associated with experimental organic pollutants
3. Identify important removal mechanisms that are relevant to environmental fate in wastewater and wetland treatment

Experimental organic pollutants included 13 fluorinated phenols and 6 named organic pollutants that represent a broad range of emerging organic pollutants, including pharmaceuticals, personal care products, and pesticides. Named organic pollutants were selected based on presence in local surface waters (Clayton County, GA) and reports of wetland removal. Chemical structures and properties of experimental organic pollutants are summarized in Table 2.5. For many of the experimental pollutants, experimental values for pollutant properties are undetermined; consequently, both estimated and experimental values are provided.

Table 2.5. Experimental organic pollutants: Structures and relevant chemical properties. Superscripts indicate: ^a From EPI Suite v. 3.10 (EPA 2002), ^b From SPARC online calculator and database (Hilal et al. 2005), and ^c Fluoxetine prescribing information (Eli Lilly 2003).

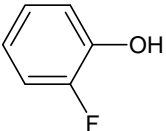
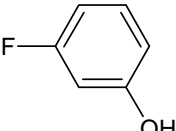
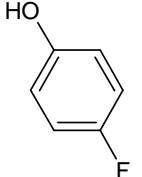
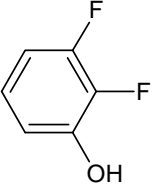
Organic pollutant (CAS #)	Structure	MW	Water solubility (mg/L)		Henry's LC (atm-m ³ /mole)		Hydrophobicity (log K _{OW})		Acid dissociation constant (pK _a)	
			Exp	Est	Exp ^a	Est ^a	Exp ^a	Est ^a	Exp ^b	Est ^b
2-fluorophenol (367-12-4)		112.1		47,439	3.22 E -6	1.86 E -6	1.71	1.71	8.70	8.59
3-fluorophenol (372-20-3)		112.1		47,439		1.86 E -6	1.93	1.71	9.21	9.25
4-fluorophenol (371-41-5)		112.1		47,439	7.05 E -7	1.86 E -6	1.77	1.71	9.46	9.47
2,3-difluorophenol (6418-38-8)		130.1		47,812		5.23 E -6		1.91		7.80

Table 2.5. (continued)

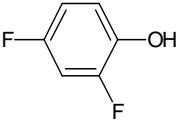
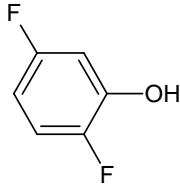
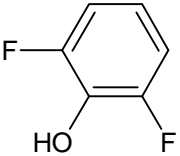
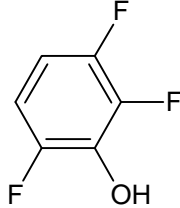
Organic pollutant (CAS #)	Structure	MW	Water solubility (mg/L)		Henry's LC (atm-m ³ /mole)		Hydrophobicity (log K _{OW})		Acid dissociation constant (pK _a)	
			Exp	Est	Exp ^a	Est ^a	Exp ^a	Est ^a	Exp ^b	Est ^b
2,4- difluorophenol (367-27-1)		130.1		47,812		5.23 E -6		1.91		8.03
2,5- difluorophenol (2713-31-7)		130.1		47,812		5.23 E -6		1.91		7.79
2,6- difluorophenol (28177-48-2)		130.1		47,812	5.23 E -6	2.43 E -5	1.96	1.91		6.98
2,3,6- trifluorophenol (113798-74-6)		148.1		47,267		1.47 E -5		2.11		6.30

Table 2.5. (continued)

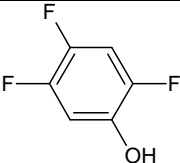
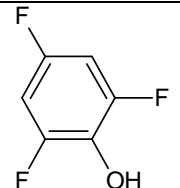
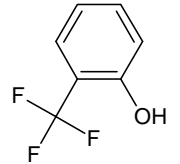
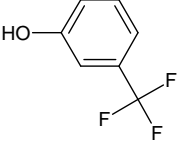
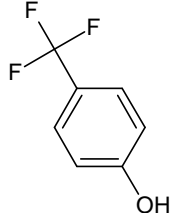
Organic pollutant (CAS #)	Structure	MW	Water solubility (mg/L)		Henry's LC (atm-m ³ /mole)		Hydrophobicity (log K _{OW})		Acid dissociation constant (pK _a)	
			Exp	Est	Exp ^a	Est ^a	Exp ^a	Est ^a	Exp ^b	Est ^b
2,4,5- trifluorophenol (2268-16-8)		148.1		47,267		1.47 E -5		2.11		7.34
2,4,6- trifluorophenol (2268-17-9)		148.1		47,267		1.47 E -5		2.11		6.53
2- trifluoromethyl- phenol (444-30-4)		162.1		1,471		4.87 E -6	2.80	2.48		8.32
3- trifluoromethyl- phenol (98-17-9)		162.1		1,471		4.87 E -6	2.95	2.48	8.95	8.77
4- trifluoromethyl- phenol (402-45-9)		162.1		1,471		4.87 E -6	2.82	2.48	8.68	7.92

Table 2.5. (continued)

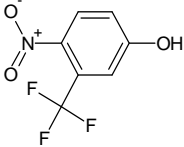
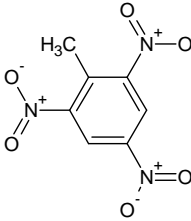
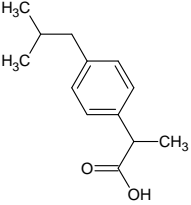
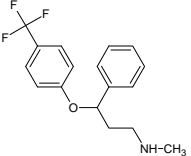
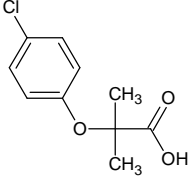
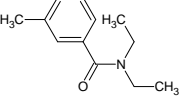
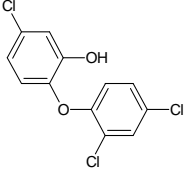
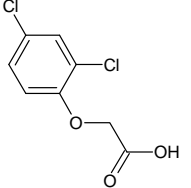
Organic pollutant (CAS #)	Structure	MW	Water solubility (mg/L)		Henry's LC (atm-m ³ /mole)		Hydrophobicity (log K _{OW})		Acid dissociation constant (pK _a)	
			Exp	Est	Exp ^a	Est ^a	Exp ^a	Est ^a	Exp ^b	Est ^b
3-trifluoromethyl-4-nitrophenol (88-30-2)		207.1	5,000	756		1.92 E -8		2.87		6.29
2,4,6-trinitrotoluene (118-96-7)		227.1	130	75.5	4.57 E -7	3.30 E -9	1.60	1.99	NA	NA
Ibuprofen (15687-27-1)		206.3	21	58.0		1.52 E -7	3.97	3.79		4.45
Fluoxetine (59333-67-4)		309.3	6,840 ^c	0.958		8.90 E -8	4.05	4.65	8.7 ^c	9.58

Table 2.5. (continued)

Organic pollutant (CAS #)	Structure	MW	Water solubility (mg/L)		Henry's LC (atm-m ³ /mole)		Hydrophobicity (log K _{OW})		Acid dissociation constant (pK _a)	
			Exp	Est	Exp ^a	Est ^a	Exp ^a	Est ^a	Exp ^b	Est ^b
Clofibric acid (882-09-7)		214.7		674		2.19 E -8	2.57	2.84		3.51
Meta-N,N-diethyltoluamide (DEET) (134-62-3)		191.3		174		2.08 E -8	2.19	2.26		1.95
Triclosan (3380-34-5)		289.6	10	9.30	2.13 E -7	3.83 E -7	4.76	4.66		7.93
2,4-dichloro-phenoxyacetic acid (94-75-7)		221.0	677	680	3.54 E -8	5.48 E-8	2.81	2.62	2.86	3.42

Fluorinated phenols

Organic chemicals commonly used in pharmaceutical, chemical, and agricultural industries are frequently halogenated. Halogenated substituents typically improve physicochemical and biochemical properties and increase lifespans of organic chemicals in environmental or human systems. Scientific awareness of potential environmental hazards of chlorinated and brominated chemicals is well established due to extensive study and examination of pollutants like perchloroethene (PCE), polychlorinated biphenyls (PCB), and polybrominated-dibenzo ethers (PBDE). Of nine chemicals classified by the European Commission as persistent organic pollutants (POPs), six are chlorinated and three are brominated. Additionally, concerns about potential environmental hazards from increased use of fluorinated synthetic chemicals are emerging.

Fluorinated synthetic chemicals are frequently used as agrochemicals and pharmaceuticals. Approximately 29% of new pharmaceuticals registered in the U.S. in 2002 were fluorinated; over 120 fluorinated compounds were registered with U.S. trade names (Bohm et al. 2004). Addition of fluorine to pharmaceutical chemicals conveys properties with medicinal benefits, but adverse effects on environmental fate. From a medicinal perspective, adding fluorine to pharmaceuticals improves metabolic stability and increases lipophilicity (Bohm et al. 2004); from an environmental fate perspective, increased stability and lipophilicity equate to decreases in degradation potential and increases in bioaccumulation potential. Fluorinated agrochemicals are also common in the U.S.; 28% of halogenated agrochemicals produced since 1940 are fluorinated (Jeschke 2004). Organic agrochemicals are fluorinated to increase toxicity, bioactivity, or lifespan in agricultural fields.

Trifluoromethylated-substituted aromatics, a subset of polyfluorinated organic pollutants that possess a trifluoromethylphenyl moiety, are used widely in agriculture,

and are gaining increased use as pharmaceuticals. Over 50% of fluorinated agrochemicals are trifluoromethyl-substituted aromatics (Key et al. 1997); most of which demonstrate pesticidal activity or toxicity (e.g., trifluralin, trifluoromethyl-nitrophenol). Additionally, many common pharmaceuticals are trifluoromethyl-substituted aromatics, including antidepressants like Prozac® and anti-inflammatory drugs like CELEBREX. Example trifluoromethyl-substituted aromatics and their uses are listed in Table 2.6.

Table 2.6. Select trifluoromethyl-substituted aromatics and their uses.

Compound	Use
Fenfluramine	Anorectic
Fluoxetine (Prozac ®)	Antidepressant
3-aminobenzotrifluoride	Pharmaceutical intermediate
Trifluralin	Herbicide
Trifluoromethyl-nitrophenol	Lampricide
Celecoxib/CELEBREX	Arthritis medication

While fluorination typically increases environmental persistence of organic pollutants, many processes can defluorinate or degrade fluorinated organic pollutants, particularly fluorophenols and trifluoromethylphenols. Important removal processes for fluorinated phenols are discussed further below. Fluorinated phenols were examined as primary experimental pollutants to expand previous research on uptake and metabolism of chlorophenols to fluorinated organic pollutants (Day and Saunders 2004; Tront et al. 2006; Tront and Saunders 2006; Tront et al. 2007; Tront and Saunders 2007).

Fluorophenols

Research on removal of fluorophenols via physicochemical processes is limited;

however, research indicated that 4-fluorophenol was photocatalytically degraded in titanium oxide solutions (Lapertot et al. 2006). Based on hydrophobicity values (i.e., log K_{OW}) provided in Table 2.5, sorption of fluorophenols to organic matter in soils or wastewaters is expected to be the predominant removal process for fluorophenols in waters. Additional removal, via volatilization, is also expected based on Henry's law constants (Table 2.5). Consequently, abiotic processes may contribute to removal of fluorophenols in wetlands and wastewater treatment.

Studies indicate potential for microbial degradation of fluorophenols in wetlands. Microbial degradation pathways for fluorophenols have been determined using ^{19}F nuclear magnetic resonance (NMR) (Boersma et al. 1998; Boersma et al. 2001). The aerobic degradation pathway in Figure 2.5 has been proposed and demonstrated for a broad suite of fluorophenols in *Rhodococcus opacus* 1G, *Rhodococcus erythropolis* 1CP, *Rhodococcus corallinus* 135, and *Rhodococcus* strain 89. Experimental *Rhodococcus* strains were isolated from both anthropogenically-impacted and non-impacted sources, including oil fields and pine forests (Boersma et al. 2001). Degradation proceeded through aromatic hydroxylation, ring cleavage by dioxygenases, conversion to fluoromuconolactones, and subsequent hydrolysis and defluorination or dehydrogenation to fluoromaleylacetate (Boersma et al. 2001). Subsequent studies utilizing fluorobenzoates indicate the fluorophenols can be completely defluorinated, yielding oxoadipic acid, in *Pseudomonas* strains (Boersma et al. 2004). Monofluorophenols, particularly 3- and 4-fluorophenol, were enzymatically oxidized by type 3 copper tyrosinase and yielded products that rapidly polymerized and released fluoride ions (Battaini et al. 2002).

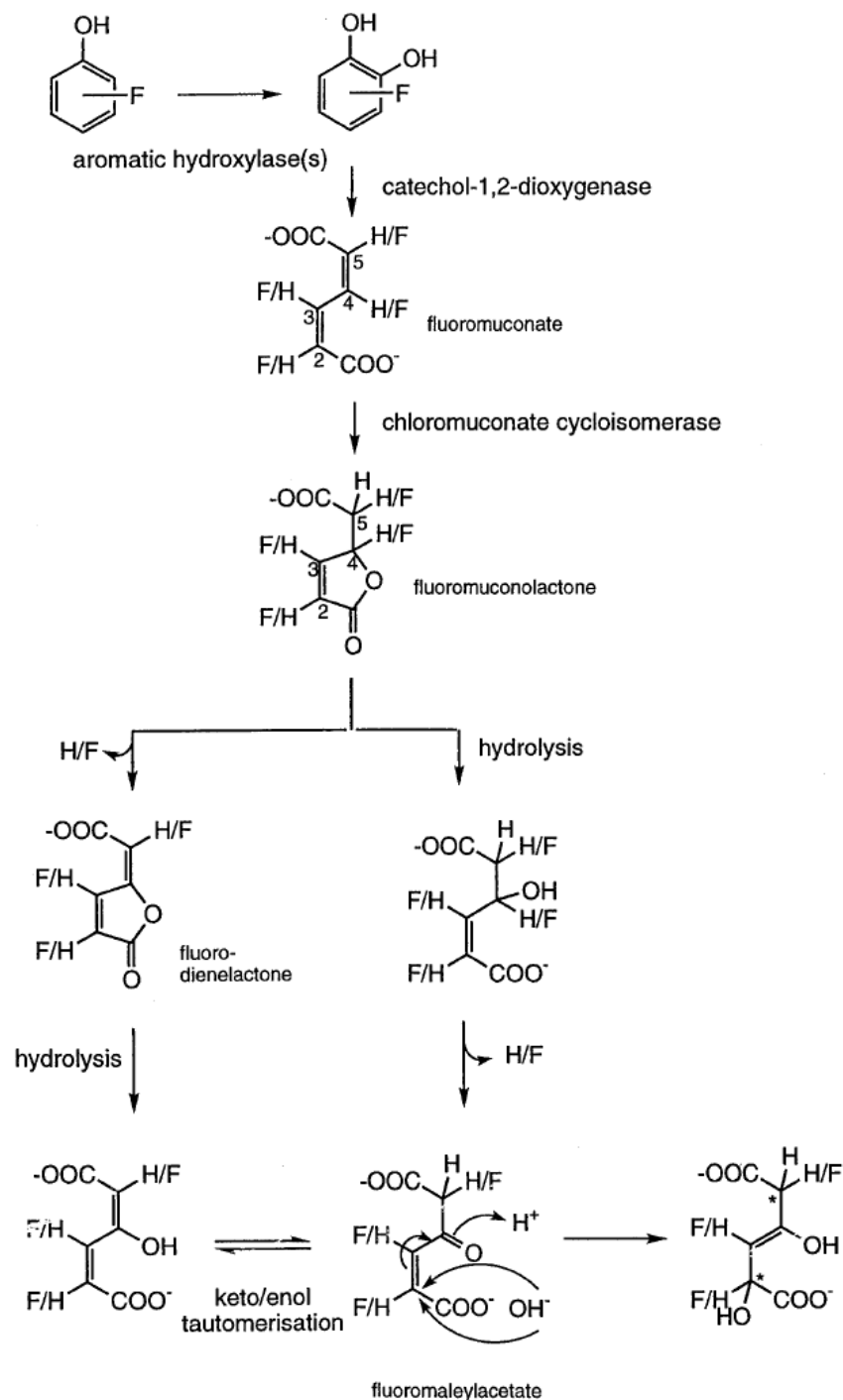


Figure 2.5. Schematic of degradation pathways for fluorinated phenols in *Rhodococcus* species from Boersma et al. (2001). Experimental fluorophenols included monofluorophenols (i.e., 2-FP, 3-FP, 4-FP), difluorophenols (i.e., 2,3-DFP, 2,4-DFP, 2,5-DFP, 2,6-DFP, 3,4-DFP, 3,5-DFP), trifluorophenols (i.e., 2,3,4-TFP, 2,3,5-TFP, 2,3,6-TFP, 2,4,5-TFP, 3,4,5-TFP), 2,3,4,5-tetrafluorophenol, and pentafluorophenol.

Trifluoromethylphenols (TFMeP)

As with fluorophenols, log K_{OW} values and Henry's law constants indicate that sorption and volatilization may contribute to removal of TFMePs in wetlands. Photolysis and hydrolysis likely also contribute to removal of TFMePs in wetlands, depending on trifluoromethyl substituent positioning. Ellis and Mabury (2000) report the hydrolysis of 2-TFMeP and 4-TFMeP to form semiquinones in aqueous solution of Borax buffer at pH 9. Release of fluoride ions was observed when 2-TFMeP and 4-TFMeP were incubated in Luria-Bertaini (LB) medium at 60 °C; increase in fluoride ions was 3 times the decrease in 2-TFMeP, indicating release of all fluorine associated with trifluoromethyl substituent (Reinscheid et al. 1998). At physiological pH of 7.4, 4-TFMeP spontaneously hydrolyzed in Krebs-HEPES buffer with 2 mg/mL glucose to form 4-hydroxybenzoic acid via a quinone methide intermediate (Thompson et al. 2000). Photolysis was observed for all mono-TFMePs when solutions were exposed to light Rayonette bulbs (365 nm) or a solar simulator. Photolytic products of 3-TFMeP included fluoride, 3-hydroxybenzoyl fluoride, and 3-hydroxybenzoic acid (Ellis and Mabury 2000).

Biotic processes may also contribute to removal of TFMePs in aquatic systems; however, studies demonstrating biological degradation are limited. Thermophilic bacteria, *Bacillus thermoleovorans* A2, degrade 2-TFMeP to 3-trifluoromethyl-catechol to 2-hydroxy-6-oxo-7,7,7-trifluorohepta-2,4-dienoate (7-TFHOD), as depicted in Figure 2.6 (Reinscheid et al. 1998). 7-TFHOD was stable in dark solutions, but rapidly decomposed in phosphate-buffer solution at pH 7.2 during exposure to sunlight.

Despite aforementioned reports of photolysis, hydrolysis, and biodegradation, TFMeP are generally considered environmentally persistent. Photolysis and hydrolysis studies were completed at pH values where >50% of TFMeP was ionized, as pK_a values for 2-TFMeP and 4-TFMeP are 8.3 and 7.9, respectively. Photolytic and hydrolytic degradation of TFMeP is likely hindered when >50% of TFMeP is protonated (e.g., pH

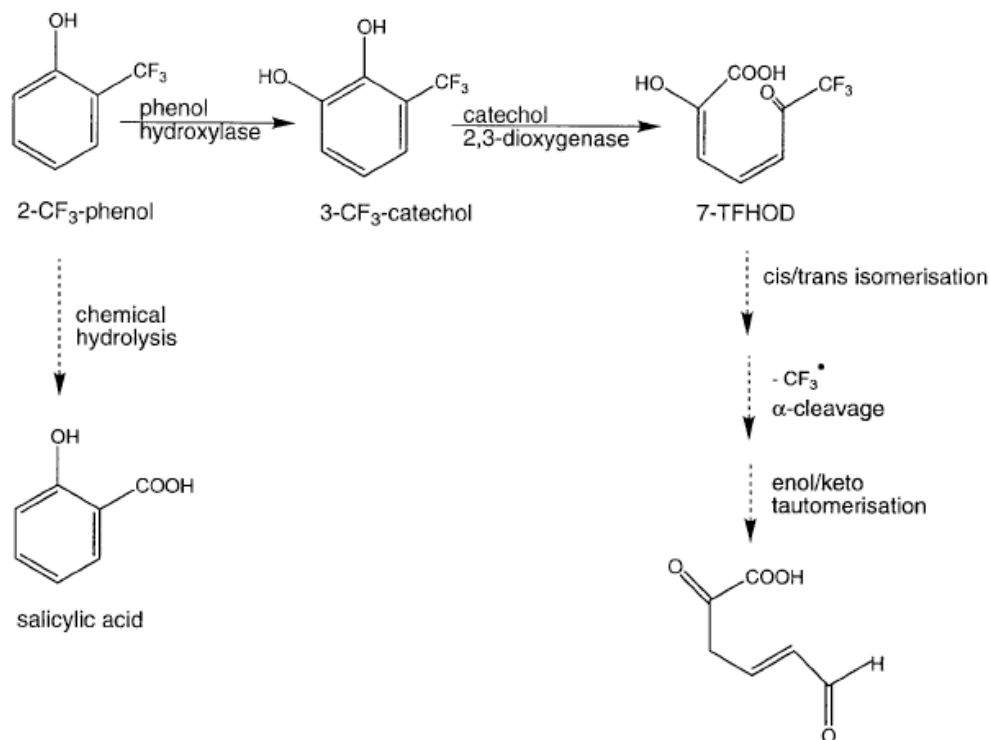


Figure 2.6. Biological (solid arrows) and abiotic (dashed arrows) degradation of 2-trifluoromethylphenol in incubations containing *Bacillus thermoleovorans* A2 and chloride-free Luria Bertani medium. Taken from Reinscheid (1998).

values ≤ 7). Furthermore, as compared to 2-TFMeP and 4-TFMeP, 3-TFMeP is extremely more persistent and is not hydrolytically or microbially degraded. Additionally, photolytic degradation of 3-TFMeP was substantially slower than for 4-TFMeP or 2-TFMeP (Ellis and Mabury 2000). Because of increased persistence of 3-TFMeP, thesis research, while looking at an entire suite of fluorinated phenols, focused on uptake and metabolism of 3-TFMeP.

Named organic pollutants

Organic pollutants, including pharmaceuticals, personal care products, and pesticides, present a growing hazard to natural resources and human and ecosystem health. Prevalence of trace concentrations of organic pollutants in U.S. surface waters is wide spread (Kolpin et al. 2002; Boyd et al. 2003; Anderson et al. 2004). Pollution of

surface water resources with bioactive pharmaceuticals and antimicrobial personal care products, such as ibuprofen, fluoxetine (Prozac®), and triclosan, prompts concerns about ecological toxicity, bioaccumulation, and human health hazards. Previous research on fate of organic pollutants in Lemnaceae systems have focused on chlorophenols and halogenated phenols, which substantially differ in structural simplicity from many pharmaceuticals, personal care products, and pesticides. Consequently, fate of named organic pollutants in Lemnaceae systems was examined to expand understanding of role of aquatic plants in wetland removal to environmentally pertinent organic pollutants. Organic pollutants were selected based on (1) presence in Clayton County surface waters, (2) aromatic chemical structure, and (3) reports of wetland removal or plant uptake. Named organic pollutants possessed a broad range of water solubility (i.e., 10 – 5,000 mg/L), Henry's law constant (i.e., 3.30×10^{-9} – 3.83×10^{-7}), log K_{OW} values (i.e., 1.60 – 4.76), and pK_a values (i.e., 1.95 – 9.58), as shown in Table 2.5.

2,4-Dichlorophenoxyacetic acid (2,4-D)

2,4-D is a broadly used pesticide that was developed during World War II to increase crop yields. Commercially released in 1946, 2,4-D is considered the first selective pesticide as 2,4-D selectively kills dicots but not monocots. Subsequently, 2,4-D is one of the most commonly used pesticides for corn, wheat, and other cereal crops (Troyer 2001). Annual usage of 2,4-D is 46 million pounds, with agricultural usage accounting for 30 million pounds (EPA 2005).

Aerobic microbial degradation of 2,4-D, yielding chlorophenols and ultimately carbon dioxide, is widely reported. In shake flasks with natural surface waters and natural sediments from Denmark, microorganisms rapidly degraded 2,4-D, with half-lives of 10 to 20 days, after a consistent lag period (Ingerslev and Nyholm 2000). At low concentrations of 2,4-D in natural waters and sediments, biodegradation was limited as microorganisms utilized 2,4-D as a secondary substrate in the presence of natural organic

matter; consequently, biodegradation rates increased with increasing concentrations of 0.2 to 2500 µg/L 2,4-D (Ingerslev and Nyholm 2000). Additionally, presence of rooted plants in unsaturated soils decreased the lag time prior to microbial mineralization of 2,4-D, indicating an important role of plants in cometabolism of 2,4-D (Shaw and Burns 2004).

Photodegradation may also contribute to 2,4-D fate. Though stable to abiotic hydrolysis, the photodegradation half life of 2,4-D in water was 7.57 days of constant light (pH 5) (EPA 2005). In laboratory studies, 2,4-D was mineralized by combinations of advanced oxidation processes, including ozonation and photocatalysis with TiO₂ (Giri et al. 2007).

3-Trifluoromethyl-4-nitrophenol (TFM)

TFM is a lampricide used in the Great Lakes basin to control invasive sea lamprey (*Petromyzon marinus*). Environmental effects of TFM, including potential estrogen agonist effects, are recognized as possibly unknown hazards (Hubert 2003). TFM is considered toxic to aquatic ecosystems. With moderate toxicity for mammals, slight to moderate toxicity for freshwater invertebrates, slight to high toxicity for freshwater fish, and high toxicity for aquatic plants, TFM can impact aquatic ecosystems at concentrations as low 1.2 mg/L TFM (Hubert 2003). However, due to the infrequency of applications (e.g., every 3 to 5 years) and selectivity of application, the EPA concluded that appropriate application of TFM does not pose unreasonable human or environmental risks (EPA 1999).

Studies typically demonstrate long-term persistence and toxicity of TFM in aqueous systems (Thingvold and Lee 1981; EPA 1999), though some studies claim that environmental effects are “transient” (Hubert 2003). There are conflicting reports on photodegradation of TFM in surface waters (EPA 1999); however, photolysis of TFM to produce trifluoroacetic acid and fluoride ions was rigorously documented (Ellis and

Mabury 2000). Microbial degradation of TFM, using microbial inoculums from activate sludge, raw sewage, and surface waters, was not observed over experimental periods of 80 d (Thingvold and Lee 1981).

Ibuprofen

Ibuprofen, which is chemically known as 2-(4-isobutylphenyl)-propionic acid, is the third most consumed drug world-wide (Buser et al. 1999). Despite limited environmental persistence, ibuprofen was detected in 9.5% of U.S. streams surveyed by USGS in 1999, with a maximum concentration of 1.0 µg/L (Kolpin et al. 2002). Based on daily consumption of 2 L drinking water for a life-span of 70 years, the average exposure to ibuprofen, via drinking water, was estimated to be 153 µg, or 0.01% of the recommended daily dose (Webb et al. 2003). Ibuprofen may adversely impact aquatic ecosystems at ppm concentrations; *L. minor* growth was inhibited by 10 µg/L ibuprofen (Pomati et al. 2004).

Ibuprofen was removed by conventional wastewater treatments systems. Studies documented greater than 90% removal of ibuprofen in activated sludge wastewater treatment systems (Ternes 1998; Buser et al. 1999; Metcalfe et al. 2003; Quintana and Reemtsma 2004). Significantly less removal (i.e., 22%) was observed in biological trickling filters in Brazil (Stumpf et al. 1999). Removal in wastewater treatment systems may be attributed to microbial degradation of ibuprofen. With 50 mg/L-wk powdered milk as carbon substrate, microorganisms isolated from activated sludge cometabolically transformed 5 mg/L ibuprofen to two isomers of hydroxy-ibuprofen (i.e., 1-hydroxy-ibuprofen and 2-hydroxy-ibuprofen) in 15 d and subsequently mineralized hydroxy-ibuprofen to non-detectable products by 22 d (Quintana et al. 2005). Carboxy-ibuprofen has also been observed as an aerobic transformation product of ibuprofen in experimental biofilm reactors (Zwiener et al. 2002). Additionally, pure microbial cultures capable of metabolic degradation of ibuprofen have been isolated from activated sludge. With

ibuprofen as the sole carbon and energy source, *Sphingomonas* species strain Ibu-2 degraded ibuprofen to catechols through removal of acidic side chains; Figure 2.7 depicts the proposed metabolic pathway for metabolic ibuprofen degradation, as indicated by detection of metabolites b to d with GC-MS (Murdoch and Hay 2005).

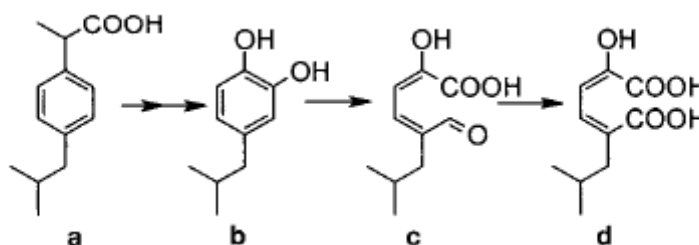


Figure 2.7. Proposed pathway for metabolic degradation of ibuprofen by *Sphingomonas* sp. strain Ibu-2. Chemical structures for ibuprofen (a) and metabolites (b – d) are provided, where b is isobutylcatechol, c is 5-formyl-2-hydroxy-7-methylocta-2,4-dienoic acid, and d is 2-hydroxy-5-isobutylhex-2,4-dienedioic acid. Taken from Murdoch and Hay (2005).

Direct photolysis of ibuprofen in Milli-Q water was not observed under sun-light or synthetic light conditions; however, slow photodegradation of ibuprofen in Mississippi River water under Hg lamps indicated that indirect, radical-mediated photolysis of ibuprofen may occur natural waters (Packer et al. 2003). With titanium oxide (TiO₂) as a catalyst, photooxidation of ibuprofen yielded ketone and alcohol via electron transfer from the carboxyl group at pH <5; ring oxidation was observed at pH >5 (Thanasawasdi et al. 2007). However, contribution of physicochemical removal processes, including photolysis, photodegradation, or photooxidation, to overall fate of ibuprofen are likely dwarfed by contributions of biological removal processes.

Fluoxetine

Marketed as Prozac® by Eli Lilly, fluoxetine is a commonly prescribed selective

serotonin reuptake inhibitor with antidepressant properties. In humans, fluoxetine is metabolized via N-demethylation to S- or R-norfluoxetine and O-dealkylation to 4-trifluoromethylphenol (Altamura et al. 1994; Liu et al. 2002). S-norfluoxetine has similar activity as fluoxetine, while R-norfluoxetine is substantially less active (Eli Lilly 2003).

Fluoxetine has been detected in U.S. surface waters at maximum concentrations of 0.012 µg/L (Kolpin et al. 2002). Concentrations as high as 0.540 µg/L fluoxetine have been detected in wastewater effluents (Westin et al. 2001). Fluoxetine exhibits ecotoxicity at ppm concentrations; consequently, standardized toxicity tests indicate minimal effects from concentrations of fluoxetine that have been detected in surface waters. Toxicity from fluoxetine was observed in *Pseudokirchneriella subcapitata* (green algae) and *Ceriodaphnia dubia* (cladoceran) at concentrations of 13.6 µg/L and 112 µg/L, respectively (Brooks et al. 2003). The effective concentration at which 50% of standard test organisms were affected (EC₅₀) ranged from 24 µg/L for *P. subcapitata* to 820 µg/L for *Daphnia magna* (Brooks et al. 2003). Concentrations ≤ 1000 µg/L fluoxetine did not adversely affect growth or health of *L. gibba* (Brain et al. 2004a), indicating that inhibition of Lemnaceae species likely does not occur at environmental concentrations.

Results on whether or not removal of fluoxetine from surface waters via photodegradation occurs are mixed. Laboratory photolysis studies have indicated that fluoxetine photodegrades to O-dealkylated products (I and II in Figure 2.8) and a potential carboxylic acid photoproduct (III in Figure 2.8) (Hubert 2003). Photodegradation occurred under simulated, high light intensity of 765 W/m² and products were identified using liquid chromatography with tandem, positive electrospray mass spectrometry. Indirect photolysis in simulated natural waters, via oxidation by hydroxyl radical, proceeded faster than direct photolysis, with a biomolecular rate constant of approximately $9.0 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ (Lam et al. 2005). The same authors also report photodegradation of 10 µM fluoxetine under natural sunlight with a half-life

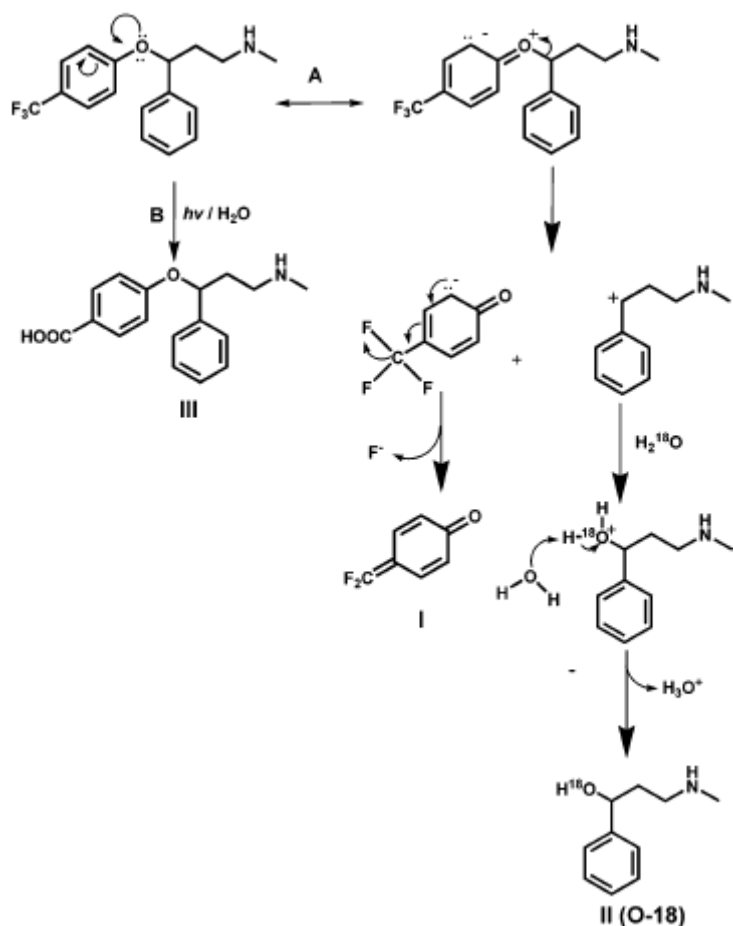


Figure 2.8. Proposed photodegradation pathway of fluoxetine leading to O-dealkylated products (I and II) and hypothesized carboxylic acid photoproduct (III). Taken from Lam et al. (2005).

of 7 ± 1 d (Hubert 2003). However, previous reports indicate that fluoxetine is stable under UV light for eight weeks (Risley and Bopp 1990). Addition of synthetic humic acids prompted photodegradation to norfluoxetine at pH 7; photodegradation did not occur in aqueous solutions, including sludge-amended solutions, that did not contain synthetic humic acids (Kwon and Armbrust 2006).

Sorption is likely an important removal process in fate of aqueous fluoxetine. In batch reactors amended with activated sludge, fluoxetine partitioned on to organic matter, but was not biologically degraded over 28 days (Kwon and Armbrust 2006). Further literature review did not discover reports of microbial degradation of fluoxetine.

Consequently, most removal observed in wastewater treatment plants is likely attributable to sorption to biosolids. Likewise, 72 to 73% of fluoxetine in sediment-water systems partitioned to sediments (Kwon and Armbrust 2006). It is important to recognize that over 94% of Prozac in human blood is irreversibly bound to human serum proteins, including albumin and α_1 -glycoprotein (Eli Lilly 2003). Thus, protein binding, in aqueous solutions containing humic acids and organic matter, may be an important component of “sorption” of fluoxetine in natural and waste- waters.

Clofibric acid

Clofibric acid, a bioactive metabolite of lipid regulating drugs like clofibrate and etofibrate, is an isomer of the pesticide mecoprop, or 2-(4-chloro-2-methylphenoxy)propionic acid. Clofibric acid has been detected in Canadian waters (Boyd et al. 2003) and German waters (Quintana and Reemtsma 2004) at relatively high concentrations of 103 ng/L and 279 ng/L, respectively; however, clofibric acid was not detected in surface waters of Louisiana (i.e., Lake Pontchartrain or Mississippi River) (Boyd et al. 2003). Clofibric acid has also been detected in tap water in Berlin, Germany at concentrations of 10 to 165 ng/L (Stan et al. 1994). Lifetime consumption of clofibric acid via drinking water consumption was estimated at 3.6 mg, or 0.7% of the daily therapeutic dose (Webb et al. 2003).

Reports of removal of clofibric acid by wastewater and water treatment processes are mixed. Removals in activated sludge wastewater treatment plants were 0% (Heberer 2002), 34% (Stumpf et al. 1999), and 51% (Ternes 1998), while removal in biological trickling filters was 15% (Stumpf et al. 1999). Sorption to biological matrixes accounted for less than 2% removal in activated sludge and membrane bio- reactors, indicating that biodegradation was responsible for removal of clofibric acid in wastewater treatment processes (Kimura et al. 2007). Additionally, clofibric acid was removed to non-detectable levels by a typical water treatment plant that utilized basic physicochemical

processes for water treatment (e.g., coagulation, flocculation, sedimentation, filtration and chlorination) (Boyd et al. 2003).

Photodegradation of clofibric acid has been observed in Milli-Q and Mississippi River water with an estimated half-life of 50 h; results indicated that direct and radical-mediated photolysis were both important mechanisms for removal of clofibric acid in sun-lit waters (Packer et al. 2003). Clofibric acid undergoes photooxidation in the presence of titanium oxides (TiO₂), yielding chloride ions from radical-mediated aromatic ring oxidation (pH>5) (Thanasawasdi et al. 2007). Mineralization of clofibric acid, via 4-chlorophenol, 4-chlorocatechol, hydroquinone, *p*-benzoquinone, and organic acid intermediates, was achieved via advanced electrochemical oxidation processes (Sires et al. 2007).

DEET

DEET, or meta-N,N-diethyltoluamide, is the active ingredient in many insect repellents. The major routes of DEET introduction into waters are (direct) application near lakes and rivers and (indirect) wastewater effluents. DEET was frequently detected agricultural, mixed, and urban lands (i.e., 85.7%, 70.0%, and 77.8%, respectively) with median concentrations of 0.036 µg/L, 0.032 µg/L, and 0.057 µg/L (Kolpin et al. 2002; Sandstrom et al. 2005). DEET concentrations in wastewater influents in Germany were as great as 0.6 µg/L in winter and spring, and as great as 3.0 µg/L in summer (Knepper 2004). Estimation of maximum exposure to DEET through drinking water consumption was 0.082 µg/kg-d, which was determined to indicate minimal risk to human health (Blanset et al. 2007).

Wastewater effluent concentrations were similar to influent concentrations in winter and spring, and near constant, at 1.0 to 1.5 µg/L, during the summer. Removal of DEET by wastewater treatment processes was only observed when influent concentrations exceeded a 1 µg/L threshold (Knepper 2004). Despite limited removal of

DEET via biological wastewater treatment processes, microbial metabolism of DEET has been reported. *Pseudomonas putida* DTB aerobically metabolized DEET to 3-methylbenzoate and diethylamine; 3-methylbenzoate was subsequently metabolized through formation of 3-methylcatechol and meta ring cleavage (Rivera-Cancel et al. 2007). The proposed pathway is illustrated in Figure 2.9.

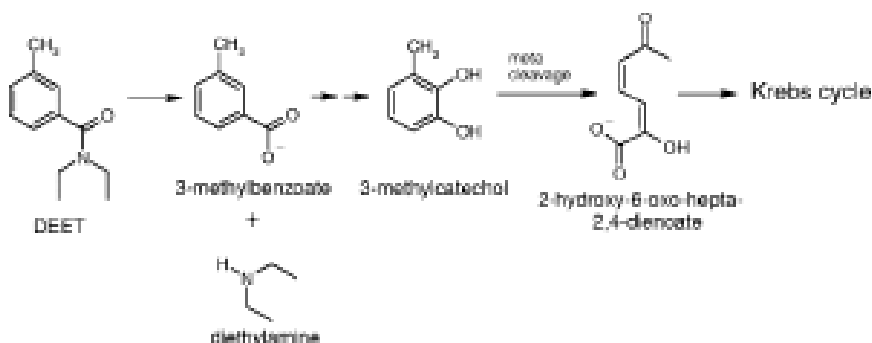


Figure 2.9. Proposed metabolic degradation of DEET by *Pseudomonas putida* DTB. Taken from (Rivera-Cancel et al. 2007).

Triclosan

Triclosan is a chlorinated antimicrobial used in many consumer products, including toothpastes and disinfecting hand gels. Triclosan was detected, with a median concentration of 0.14 µg/L, in 57.6% of U.S. streams surveyed by USGS in 1999 (Kolpin et al. 2002). Concentrations of triclosan in Louisiana municipal wastewater treatment plants were 10 to 20 ng/L (Boyd et al. 2003). Higher concentrations of triclosan, ranging from 40 ng/L to 213 ng/L were observed in wastewater treatment plant effluents in Switzerland (Singer et al. 2002).

As compared with many pharmaceuticals or personal care products, evidence of adverse environmental and human health effects from triclosan has been established.

Environmentally relevant concentrations of triclosan (i.e., 0.03 to 0.15 $\mu\text{g/L}$) disrupt thyroid-associated gene expression in American bullfrog, with noticeable effects on tadpole weight at concentrations as low as 0.15 $\mu\text{g/L}$ (Veldhoen et al. 2006). Triclosan has been detected in human plasma at concentrations ranging from 0.018 ng/g to 38 ng/g (Allmyr et al. 2006). Although concentrations were substantially higher in individuals using personal care products with triclosan (i.e., median concentration of 16 ng/g), accumulation of triclosan was also observed in individuals not using triclosan-containing products (i.e., median concentration of 0.072 ng/g), indicating that drinking water may be an alternative source of exposure. The maximum estimated exposure to triclosan, via drinking water consumption, was estimated to be 0.193 $\mu\text{g/kg/d}$ (Blanset et al. 2007).

Fate of triclosan in wastewater treatment systems and natural waters has been previously described. Removal in wastewater treatment plants were attributed to biological degradation (79%) and sorption to activated sludge (15%); only 6% of influent triclosan was detected in wastewater treatment plant effluents (Singer et al. 2002). Similarly high removal rates of triclosan have been observed for rotating biological contactors (58 to 96%), trickling filters (86 to 97%), and activated sludge systems (95 to 98%) (Thompson et al. 2005). Studies utilizing radiolabeled triclosan indicated that triclosan is mineralized to carbon dioxide in activated sludge systems; however, biodegradation pathway for triclosan was not proposed (Federle et al. 2002). In natural waters and treatment systems, photodegradation can account for up to 80% of removal (Tixier et al. 2002). Photodegradation of triclosan yields monochlorophenols, dichlorophenols, 2,8-dichlorodibenzo-*p*-dioxin, and oligomerization products (Latch et al. 2005; Sanchez-Prado et al. 2006). The proposed pathway for photodegradation of triclosan is presented in Figure 2.10. Production of toxic and carcinogenic chlorinated dioxins and furans prompts additional concerns about the environmental fate of triclosan. Removal of triclosan in aquatic systems is also mediated by presence of manganese oxides, which can rapidly oxidize triclosan to *p*-(hydro)quinine products (Zhang and

Huang 2003).

Previous research with fluorinated phenols and named organic pollutants indicates that many removal processes may contribute to fate of organic pollutants in wetland systems. Photodegradation, sorption to organic matter, and microbial degradation are three prominent examples of removal processes that occur with or without the presence of plants in wetlands. However, it is important to note that Lemnaceae growth in wetlands will affect all three processes. Dense Lemnaceae growth can limit photodegradation while providing ample organic matter for sorption of organic pollutants. Lemnaceae may also enhance microbial degradation of organic pollutants by providing carbon substrates to enhance microbial degradation and prompting cometabolic degradation. Consequently, even while excluding active plant uptake and metabolism, Lemnaceae may play an important role in fate of organic pollutants in wetlands.

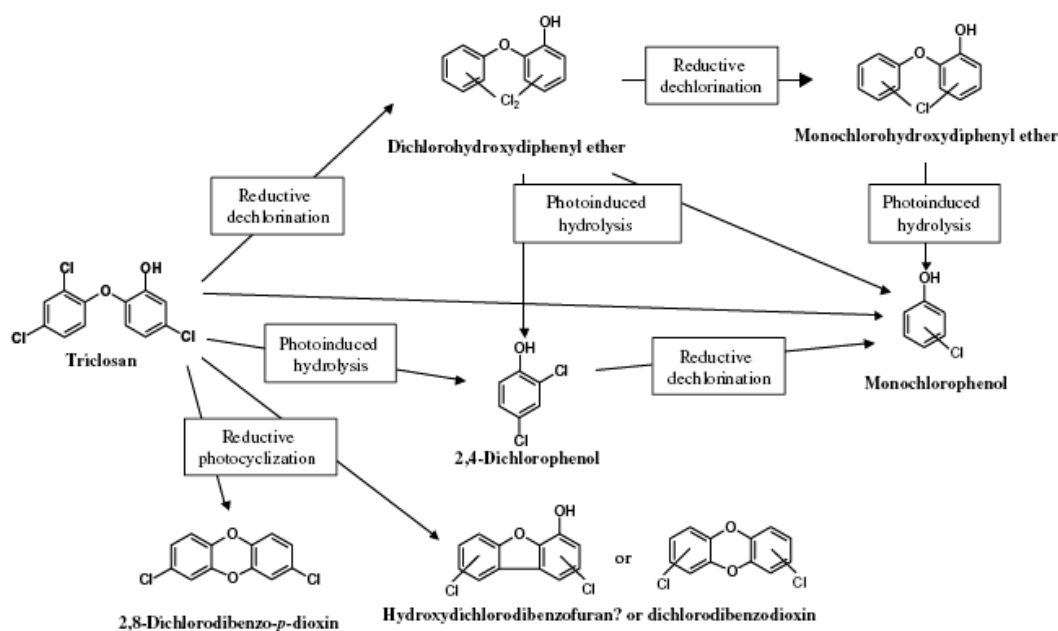


Figure 2.10. Proposed photodegradation pathway of triclosan in waters. Taken from (Sanchez-Prado et al. 2006).

For many organic pollutants, particularly fluorinated phenols, plant uptake is expected to play a primary role in fate of organic pollutants in wetlands. Consequently, research on effects of experimental and environmental parameters on uptake by plants is needed. Research presented in this thesis utilizes controlled batch systems with Lemnaceae to examine dependence of plant uptake on pollutant concentration, pollutant properties, and air temperature. Additionally, thesis research broadens scientific understanding of the role of plant uptake in fate of organic pollutants by expanding uptake studies to include fluorinated phenols and named organic pollutants with a broad range of chemical properties and uses (e.g., pharmaceuticals, personal care products, and pesticides).

CHAPTER 3: UPTAKE OF FLUORINATED PHENOLS BY LEMNACEAE

Introduction

Fluorinated organic chemicals, which are commonly used in agriculture, industry, and medicine, are emerging as a new class of bioaccumulative and persistent pollutants. Use of fluorinated chemicals is prevalent and increasing; for example, 28% of halogenated agrochemicals produced since 1940 are fluorinated (Jeschke 2004). Over 50% of fluorinated agrochemicals are trifluoromethyl-substituted organic chemicals, including the common pesticide trifluralin (Key et al. 1997; Jeschke 2004). Many fluorinated and trifluoromethyl-substituted agrochemicals are recognized hazards. For example, 3-trifluoromethyl-4-nitrophenol (TFM), a lampricide used in the Great Lakes basin, is an estrogen agonist that possesses an endocrine disruptor effect (EPA 1999), while 3-aminobenzotrifluoride is a known human toxin. Likewise, bioactive fluorinated pharmaceuticals may adversely affect ecosystems. With increasing use of fluorinated organic chemicals as agrochemicals and pharmaceuticals, it is important to investigate the fate of fluorinated organic pollutants in both natural and engineered aquatic systems.

In natural and engineered systems, aquatic plants actively remediate surface waters and wastewaters. Engineered wetlands and duckweed lagoon systems are commonly used in both rural and urban settings to polish nutrients from agricultural runoff and to treat secondary wastewaters (Cheng et al. 2002; Cameron et al. 2003; Zimmo et al. 2004). As integral components of natural ecosystems, aquatic plants assimilate chemicals that are characteristic of both non-point and point pollutants (Wang et al. 2002; Williams 2002). Despite established role of aquatic plants in environmental fate and treatment of aqueous nutrients, the interactions between aquatic plants and organic pollutants are not well characterized. Quantitative relationships that describe

uptake of emerging organic pollutants by aquatic plants are needed to better understand and design systems to phytoremediate polluted surface waters.

Uptake of organic pollutants by aquatic plants is typically characterized by pseudo-first-order rate coefficients. Studies have indicated that pseudo-first-order uptake rate coefficients are dependent upon plant mass and initial chemical concentration (McCutcheon et al. 2003). In contrast, uptake of organic pollutants by terrestrial plants is typically described by an equilibrium concentration factor, such as transpiration stream concentration factors (TSCF) and root concentration factors (RCF). Terrestrial plant concentration factors are measured as the ratio of the concentration of chemical in a specified portion of the plant to the concentration in the external media. Studies have indicated that uptake of organic pollutants by terrestrial plants, as quantified by TSCF and RCF, depended on chemical parameters that described organic partitioning, such as the octanol-water partitioning coefficient, or $\log K_{OW}$ (Briggs et al. 1982; Burken and Schnoor 1998). However, relationships that have been previously developed for terrestrial plant concentration factors are not necessarily applicable to aquatic plant uptake rate coefficients. For example, uptake of halogenated phenols by *L. minor* was independent of $\log K_{OW}$ (Tront et al. 2007), indicating that partitioning of halogenated phenols into *L. minor* did not control uptake.

Uptake of organic pollutants by plants is typically described as a complex series of processes. Through sorption, diffusion, partitioning, and water translocation, aqueous contaminants accumulate inside plant cells. Continued uptake depends on plant metabolism of these internal organic contaminants – a serial chain of biochemical reactions that includes transformation of parent pollutants, conjugation of metabolites with macromolecules, and incorporation of conjugated products into cell walls and vacuoles (McCutcheon and Schnoor 2003). The uptake of 2,4-dichlorophenol (2,4-DCP) by aquatic plants, particularly *L. minor*, has been extensively studied (Day and Saunders 2004; Pascal-Lorber et al. 2004) and involves many processes typical of uptake of

organic pollutants by plants, including abiotic partitioning, conjugation of 2,4-DCP with carbohydrates (i.e., glycosylation), and sequestration into plant tissue (Figure 2.4). In sum, uptake of organic pollutants by Lemnaceae relies on a complex combination of abiotic and plant-driven processes. Therefore, quantitative relationships that describe uptake of organic pollutants by aquatic plants must consider both abiotic and plant-driven processes.

Improving design of aquatic plant systems to remediate surface waters requires a better understanding of agrochemical uptake by aquatic plants. Objectives of this study were to evaluate uptake of fluorinated organic pollutants by Lemnaceae and to attempt to develop relationships between uptake rate coefficients and chemical properties.

Examination of a subset of fluorinated phenols (i.e., fluorophenols and trifluoromethylphenols) allowed for observations of factors affecting various uptake processes and evaluation of previously determined relationships for uptake. Based on previous studies, it was predicted that enzymatic processing was the rate-limiting step of uptake. Therefore, chemical properties related to enzymatic processing, such as substituent position and type, was expected to be more related to uptake rates than chemical properties related to partitioning, such as log K_{OW} or acid dissociation constants (pK_a).

Material and Methods

Collection and Maintenance of Lemnaceae

Lemnaceae, identified as *Lemna minor*, was collected from a tertiary wastewater-reclamation wetland located near Atlanta, GA and operated by the Clayton County Water Authority. The wetland is used to polish secondary wastewaters prior to discharge into the raw-water reservoir for the potable-water system of Clayton County, GA. Stock Lemnaceae was maintained under simulated natural conditions in laboratory tanks with

nutrients supplied from or wetland detritus. Lemnaceae was cultured under an 18-h photoperiod in a growth chamber at 22°C. Prior to experiments, *L. minor* was thoroughly rinsed with distilled water and weighed, where mass was determined after removal of excess water through a 10-min period of air-drying and blotting with paper towels.

Uptake of Fluorinated Phenols by *L. minor*

Batch experiments were used to examine effects of chemical structure and related physicochemical properties on uptake rates of fluorinated phenols. *L. minor* was exposed to 14 structurally similar fluorophenols and trifluoromethylphenols for 50 h. Three monofluorophenols (FP), three monotrifluoromethylphenols (TFMeP), four difluorophenols (DFP), three trifluorophenols (TFP), and 3-trifluoromethyl-4-nitrophenol (TFM) were selected as experimental pollutants. For comparison with TFM, uptake of 2,4,6-trinitrotoluene (TNT) was also determined. Experimental organic pollutants were chosen to permit examination of a suite of similar compounds possessing and substantial range of chemical properties.

Uptake of fluorophenols and trifluoromethylphenols was quantified in batch reactors containing 10 µM fluorophenol or trifluoromethylphenol, 1.0 g *L. minor*, and 100 mL of Standard Methods medium (APHA et al. 1998b). To mimic growth observed in natural and treatment wetlands, high plant density of 1.0 g per 100 mL (approximately 420 g/m²) was used. Medium pH was buffered to pH 5.0 or 7.0 using phosphate buffer. As previous studies have indicated that uptake rates are dependent on the fraction of 2,4-DCP protonated in the external aqueous phase (Tront and Saunders 2006), medium pH was chosen to guarantee that experimental organic pollutants were >90% protonated in aqueous medium. Aqueous-phase concentrations were sampled over 50 h. Since previous studies have indicated that almost 100% of 2,4,5-trichlorophenol is removed in 50 h (Tront and Saunders 2006), an exposure period of 50 h was chosen to allow for a short-term determination of uptake. Additionally, plant growth was negligible over 50 h

(0.06 ± 0.08 g per 1.0 g *L. minor*), allowing for elimination of changing plant mass as a variable in uptake. Samples were analyzed on an Agilent (Palo Alto, CA, USA) Model 1100 liquid chromatography (LC) system equipped with a UV/Vis diode array detector (DAD) and mass spectrometer (MS). Fluorinated phenols were separated from medium components using isocratic separation with acetonitrile (40%) and 0.1% acetic acid in water (60%) on a reverse phase STABLE BOND-C18 analytical column (2.1x150 mm, 5 μ m, Agilent). Quantification of fluorinated phenols were completed at a wavelength of 210 nm. The absence of aqueous metabolites was monitored using DAD at 210 nm and electrospray ionization (ESI) in negative mode on MS.

Oxygen production rates (μ mol O₂/h-g *L. minor*) were quantified at the conclusion of uptake measurements to determine if *L. minor* was inhibited by fluorinated phenol exposure. The oxygen production rate assessment has been described previously (Reinhold and Saunders 2006a; Tront and Saunders 2006), and utilized sealed reactors containing 0.5 g *L. minor* and 60 mL medium. After 24 h, the headspace volume was measured with manometers and the partial pressure of oxygen was quantified on a gas chromatograph equipped with a thermal conductivity detector (GC-TCD) and molecular sieve column. Oxygen production rates for exposed and non-exposed *L. minor* were compared to determine if *L. minor* was inhibited during uptake measurements.

Microbial degradation and sorption controls were conducted for all experimental fluorinated phenols. To account for microbial degradation and sorption of fluorinated phenols, *L. minor* was inactivated prior to experimentation through exposure to darkness in sealed reactors for >12 h. Oxygen production rate assessments indicated that photosynthetic activity ceased in sealed, dark reactors with *L. minor* after 4 h (example provided in Figures 5.1). After introduction of 10 μ M fluorinated phenol, reactors were kept in darkness during the 50-h exposure and measurement period. Since 2-trifluoromethylphenol and 4-trifluoromethylphenol are reported to undergo photolysis (Ellis and Mabury 2000), reactors containing medium and 10 μ M trifluoromethylphenol

were used to determine if photolytic degradation of trifluoromethylphenols was possible in the experimental reactors. However, it is important to note that experimental design incorporated full surface coverage of *L. minor* and coverage of reactor sidewalls, minimizing light inputs into experimental reactors.

Statistical Analyses

Uptake rates coefficients were determined from aqueous concentration data. Aqueous concentrations over time were normalized to initial concentration measurements (i.e., C_I = concentration determined for sample taken at $t_I=10$ min following addition of plants to the medium). Sorption studies have indicated that rapid equilibration occurs in the first 10 min of exposure to inactivated *L. minor* (Tront and Saunders 2006). Therefore, normalization to C_I samples accounted for equilibration and for water additions made with addition of plants to reactors. Uptake rate coefficients were determined by fitting normalized concentration data with 2-parameter, first-order exponential decay relationships with Sigma Plot 2000 (Systat Software Inc. 2000). If fit of normalized concentration data did not pass statistical validity criteria ($r^2>0.90$ and $p<0.01$), the uptake rate coefficient was deemed to be zero.

Potential relationships between physicochemical properties of fluorinated phenols and uptake by *L. minor* were investigated. Experimental values were used when available (Howard and Meylan 1997). Theoretical K_{OW} values were calculated with the KOWWIN program (EPA 2002). Acid dissociation constants (pK_a) were determined by SPARC online calculator (Hilal et al. 2005) and ACD software (ACD/Lab 2005). Hammett constants (σ) were determined with established methodology (Hansch and Leo 1995). Hammett's constants are used to describe electron withdrawing or donating nature of substituents and are frequently used to develop relationships for interactions between organic pollutants and enzymes.

Results and Discussion

Uptake of Fluorinated Phenols by *L. minor*

Fluorophenols and trifluoromethylphenols were rapidly removed from aqueous medium by *L. minor*. Decreases in aqueous-phase concentrations were attributed to plant-driven uptake, as controls indicated negligible microbial degradation and physicochemical losses in the experimental reactors. No change in concentration was observed in independent control reactors containing trifluoromethylphenols that did not contain *L. minor*, indicating negligible losses from physicochemical processes, including photolytic degradation and volatilization. Loss of fluorinated phenols in dark reactors with inactivated *L. minor* was limited to an initial decrease in the first ten minutes that was attributed to sorption. Statistically similar initial decreases were observed in experimental reactors and were accounted for in uptake rate calculations by normalization of concentration to concentration at 10 min (C_1). Subsequent loss of fluorinated phenols was not observed in the microbial controls, indicating an absence of microbial degradation in experimental reactors. Additionally, oxygen production rates were statistically similar for exposed *L. minor* (i.e., 13.0 ± 2.7 to 16.6 ± 1.9 $\mu\text{mol O}_2/\text{h-g}$) and for non-exposed *L. minor* (i.e., 14.8 ± 0.7 $\mu\text{mol O}_2/\text{h-g}$), indicating that that initial concentrations (10 μM) did not inhibit *L. minor* activity.

Aqueous removal typically followed first-order exponential decay relationships, as shown in Figure 3.1. Pseudo-first-order uptake rate coefficients for fluorophenols and trifluoromethylphenols ranged from 0.20 ± 0.04 d^{-1} to 0.84 ± 0.07 d^{-1} , suggesting rapid aqueous depletion of fluorophenols and trifluoromethylphenols by aquatic plants in naturally vegetated and engineered wetland systems.

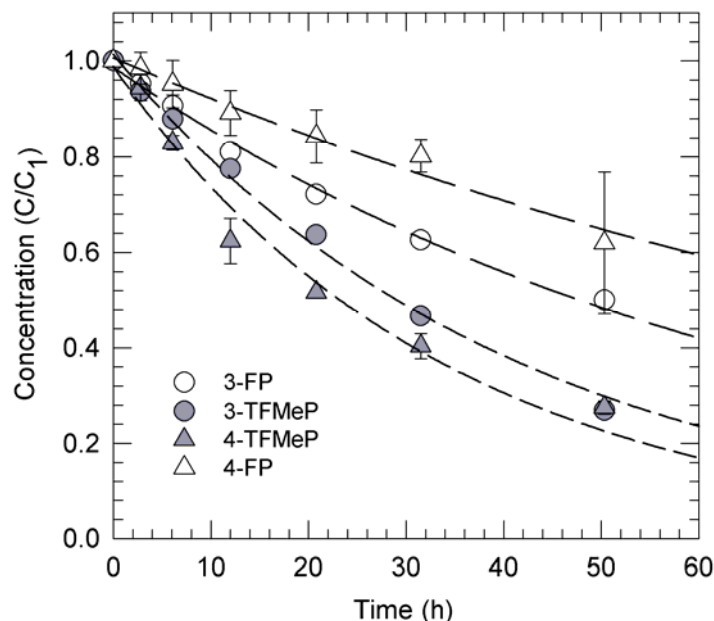


Figure 3.1. Normalized aqueous concentration of 3-fluorophenol (3-FP), 3-trifluoromethylphenol (3-TFMeP), 4-trifluoromethylphenol (4-TFMeP), and 4-fluorophenol (4-FP) with time in batch reactors with 0.5 g *L. minor*. Error bars represent standard error for triplicate reactors. Dashed lines are fitted first-order exponential decay relationships. Fitted pseudo-first order uptake rate coefficients were $0.34 \pm 0.01 \text{ d}^{-1}$ for 3-FP, $0.58 \pm 0.02 \text{ d}^{-1}$ for 3-TFMeP, $0.71 \pm 0.03 \text{ d}^{-1}$ for 4-TFMeP, and $0.21 \pm 0.03 \text{ d}^{-1}$ for 4-FP.

Effects of Fluorinated Substituents

Further examination of uptake rate coefficients for monofluorophenols and monotrifluoromethylphenols indicated that uptake of trifluoromethylphenols by *L. minor* was statistically similar to, or greater than, uptake of fluorophenols (Figure 3.2). Uptake rate coefficients of 2-TFMeP and 2-FP were statistically similar (i.e., $0.39 \pm 0.02 \text{ d}^{-1}$ vs. $0.44 \pm 0.03 \text{ d}^{-1}$), while uptake rate coefficients for 3-TFMeP and 4-TFMeP were greater than those for 3-FP and 4-FP (i.e., $0.58 \pm 0.02 \text{ d}^{-1}$ and $0.71 \pm 0.03 \text{ d}^{-1}$ vs. $0.34 \pm 0.01 \text{ d}^{-1}$ and $0.21 \pm 0.03 \text{ d}^{-1}$, respectively). It is important to note that no photolytic degradation products were observed in aqueous solution and no loss of 4-TFMeP was observed in experimental reactors systems without *L. minor*. Consequently, aqueous removal was a

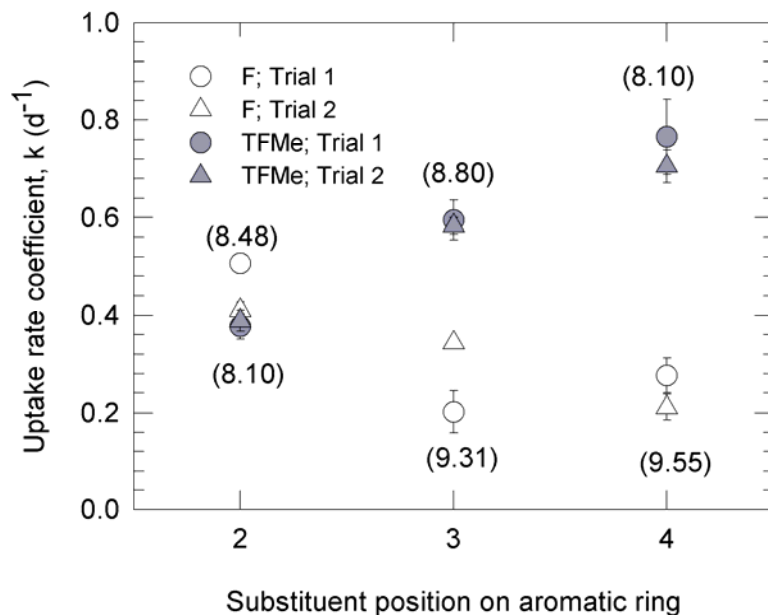


Figure 3.2. Uptake rate coefficients for fluorinated phenols based on position of fluoro (F) and trifluoromethyl (TFMe) substituents determined through two experiments (Trial 1 and Trial 2). Numbers in parentheses are estimated pK_a values. Error bars represent standard error of determination for composite data from triplicate reactors.

plant-driven process and differences in pseudo-first order rate coefficients resulted from unidentified characteristics of the interactions between fluorinated phenols and *L. minor*.

The observed trend of more rapid uptake of TFMeP than FP was hypothesized to relate to greater electronegativity of the trifluoromethyl substituent and its effects on enzymatic processing of fluorinated phenols by *L. minor*. Glucosylation, or enzymatic addition of a carbohydrate to an organic chemical, is the first enzymatic process in the metabolism of 2,4-DCP and other halogenated phenols by *L. minor* (Ensley et al. 1997; Day and Saunders 2004; Pascal-Lorber et al. 2004). It was assumed that similar metabolic pathways exist for fluorinated phenols studied herein. After abiotic partitioning of halogenated phenols in plant cells, halogenated phenols must deprotonate before glucosylation can occur. Deprotonation may occur either from acid-base equilibrium within the plant cytosol or through action of glucosyltransferases prior to

transfer of glucose to the halogenated phenol. In either case, availability of an easily-deprotonated hydroxyl group could affect rates of enzymatic processing.

In the *meta* and *para* positions, the trifluoromethyl group (TFMe) would more greatly affect stability of the hydroxyl group, as evidenced by lower acid-base dissociation constants (i.e., $pK_{a,3-TFMe}=8.8$ vs. $pK_{a,3-F}=9.3$, $pK_{a,4-TFMe}=8.1$ vs. $pK_{a,4-F}=9.6$). In the *ortho* position, the effect of trifluoromethyl group on the hydroxyl group is more subtle, and the difference between pK_a of 2-TFMeP and 2-FP is lower (i.e., $pK_{a,2-TF}=8.1$ vs. $pK_{a,2-F}=8.5$). Additionally, the trifluoromethyl group in the *ortho* position may negatively affect enzymatic glucosylation through negative proximity effects, including hydrogen bonding between the trifluoromethyl group and the hydroxyl group (Kovacs et al. 1996) or steric hindrance of glucosyltransferases responsible for conjugating halogenated phenols. In other words, the trifluoromethyl group in the *ortho* position may interfere with the ability of glucosyltransferases to interact with 2-TFMeP. Consequently, variations in uptake rate coefficients for trifluoromethylphenols and fluorophenols were attributed to availability and accessibility of the ionized hydroxyl group to the glucosyltransferase enzyme.

A positive, linear correlation was observed between uptake rate coefficients (k) and Hammett's constant (σ) for monofluorophenols, where $k\text{ (d}^{-1}\text{)} = 0.19 + 0.41\sigma$ (i.e., $r^2=0.997$, $p=0.05$). These results indicated that differences in uptake rate coefficients might be attributed to differences in enzymatic rates, as similar relationships have been observed for mono-substituted phenols interactions with enzymes. For example, activity of tyrosinase toward monofluorophenols was dependent on Hammett's constant, σ (Battaini et al. 2002). Additionally, activity of a glycosyltransferase isolated from pig liver towards *para*-substituted phenols increased linearly with increasing σ (Magdalou et al. 1982). However, both these relationships, and the one found in this study, were confined to rigidly defined classes of organic pollutants with few compounds (e.g., 3 monofluorophenols and 10 *para*-substituted phenols). Furthermore, a similar relationship

was not observed for trifluoromethylphenols, difluorophenols, or trifluorophenols. Consequently, while both uptake rates and enzymatic transformation rates of monofluorophenols were correlated with σ , these results solely indicate the importance of considering enzymatic transformation rates in uptake of organic pollutants by *L. minor*. Hammett's constants (σ) were not indicative of patterns of uptake rates.

The effect of substituent positioning on uptake rate coefficients was examined with eight ortho-substituted fluorophenols. Uptake rate coefficients ranged from 0.26 to 0.84 d⁻¹ (Figure 3.3). Uptake rate coefficients of difluorophenols (i.e., 0.27±0.04 – 0.65±0.03 d⁻¹) and trifluorophenols (i.e., 0.30±0.04 – 0.84±0.07 d⁻¹) bracketed the uptake rate coefficient of 2-FP (i.e., 0.44±0.03 d⁻¹), indicating independence of uptake rate coefficients on number of fluorine substituents. Results further supported the hypothesis that substituent position affects uptake rate coefficients. Fluorine substitution in both ortho- positions (i.e., 2,6-DFP, 2,3,6-TFP, and 2,4,6-TFP) hindered uptake, as evidenced by uptake rate coefficients of 0.27±0.04 d⁻¹, 0.37±0.05 d⁻¹, and 0.30±0.04 d⁻¹,

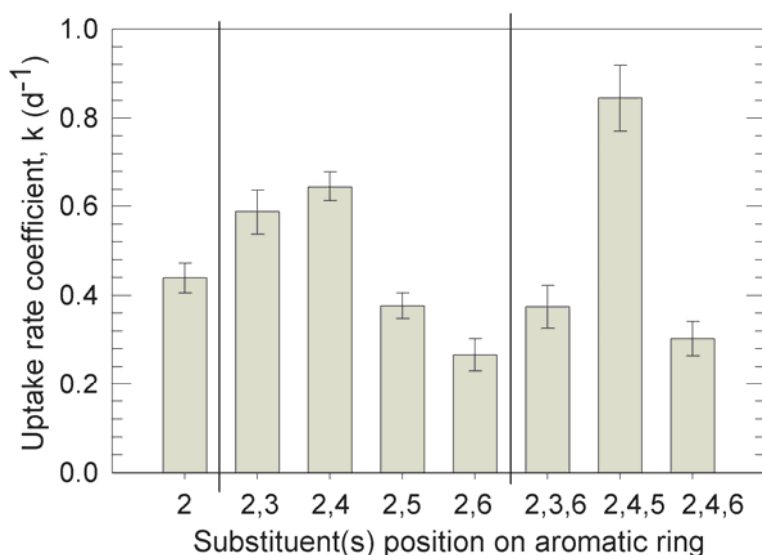


Figure 3.3. Uptake rate coefficients for fluorinated phenols based on substituent position. Error bars represent standard errors of determination for composite analysis of triplicate reactors.

respectively. However, this explanation is not adequate for hindered uptake of 2,5-DFP (i.e., $0.34 \pm 0.04 \text{ d}^{-1}$), illustrating the complex nature of uptake of fluorinated phenols by *L. minor*.

Hindered uptake of *ortho*-, *ortho*-substituted fluorophenols was attributed to proximity effects of fluorophenols on glucosyltransferase enzymes. As glucosylation of fluorophenols requires approach of the fluorophenol to within bonding distance of activated glucose and the enzyme, a negative proximity effect was considered a probable explanation for decreased uptake rates of *ortho*-, *ortho*-substituted fluorophenols. Two potential proximity effects were considered: steric hindrance and repulsion between the *ortho*-fluorine and the enzymatic reaction site. Studies utilizing a glucosyltransferase from *Sorghum bicolor* (UGT85B1) have indicated that UGT85B1 activity required availability of sterically unhindered primary hydroxyl group (Meßner et al. 2003). An alternative explanation for the hypothesized hindrance of enzymatic processing of *ortho*-, *ortho*-substituted fluorophenols was interference of the *ortho*-, *ortho*-fluorine with amino acids of the enzyme active site. Glucosyltransferases contain a conserved portion, an Asp/Glu residue that is responsible for deprotonation of substrates, that could electronically repel the *ortho*-substituted fluorine, depending on the spatial arrangement of the active site, glucose, and fluorinated phenol.

The greatest uptake rate coefficients for fluorinated and halogenated phenols were for 2,4,5-trifluorophenol (2,4,5-TFP, $k=0.84 \pm 0.07 \text{ d}^{-1}$; this study) and 2,4,5-trichlorophenol (2,4,5-TCP, $k=1.03 \pm 0.11 \text{ d}^{-1}$; (Tront et al. 2007)). Other studies have observed increased enzymatic activity toward 2,4,5-TCP in plants, indicating that the elevated uptake rate coefficients for 2,4,5-TFP and 2,4,5-TCP may be related to increased enzymatic activity. For example, glucosyltransferase activity in *Arabidopsis thaliana* was elevated for 2,4,5-TCP when compared to other substrates, including pentachlorophenol (Meßner et al. 2003). Furthermore, studies have concluded that plants contain multiple, overlapping isoenzymes that are responsible for substrate-specific

glycosylation (Schaffner et al. 2002). While these studies did not examine the fluorinated organics presented herein, they collectively indicate that different glucosyltransferases may be responsible for transformation of structurally similar fluorinated phenols. Consequently, uptake rates of fluorinated phenols by *L. minor* may depend on a multitude of uncharacterized enzymatic reactions and affinities. The existence of multiple isoenzymes acting upon different fluorinated phenols would account for the relationship between Hammett's constants and uptake rate coefficients observed for monofluorophenols, but not for di- or trifluorophenols.

Effects of Partitioning-Related Properties

Previous experiments had indicated a tentative, positive correlation between pseudo-first-order uptake rate coefficients and fraction of halogenated phenol protonated internal to the plant cell (i.e., $r^2=0.66$) (Tront et al. 2007). Results for uptake of fluorinated phenols (this study) indicated that a similar relationship was inappropriate for fluorinated phenols, as uptake rate coefficients did not depend on fraction of fluorinated phenol protonated at cytosolic pH (i.e., $r^2 = 0.07$ and $p=0.36$; Figure 3.4). Uptake rate coefficients for fluorinated phenols were independent of fraction protonated at cytosolic pH whether fraction protonated was calculated from experimental or estimated (i.e., SPARC and ACD) pK_a values.

It is important to consider uncertainties inherent to relationships that rely on fraction of halogenated phenol protonated in the plant cytosol. Of thirteen fluorinated phenols studied herein, experimental pK_a -values were only published for five (Mackay et al. 1997). SPARC and ACD estimations of pK_a varied by as much as 0.90 pH-units and errors associated with ACD estimations were as large as 0.23 pH-units. In respect to fraction protonated at pH 7, these errors were substantial enough to hinder analysis; for example the ACD-estimated pK_a of 2,4,6-TFP is 7.47 ± 0.23 which yields a fraction protonated at pH 7.0 of 0.72 ± 0.09 . Consequently, the relationship between uptake rate

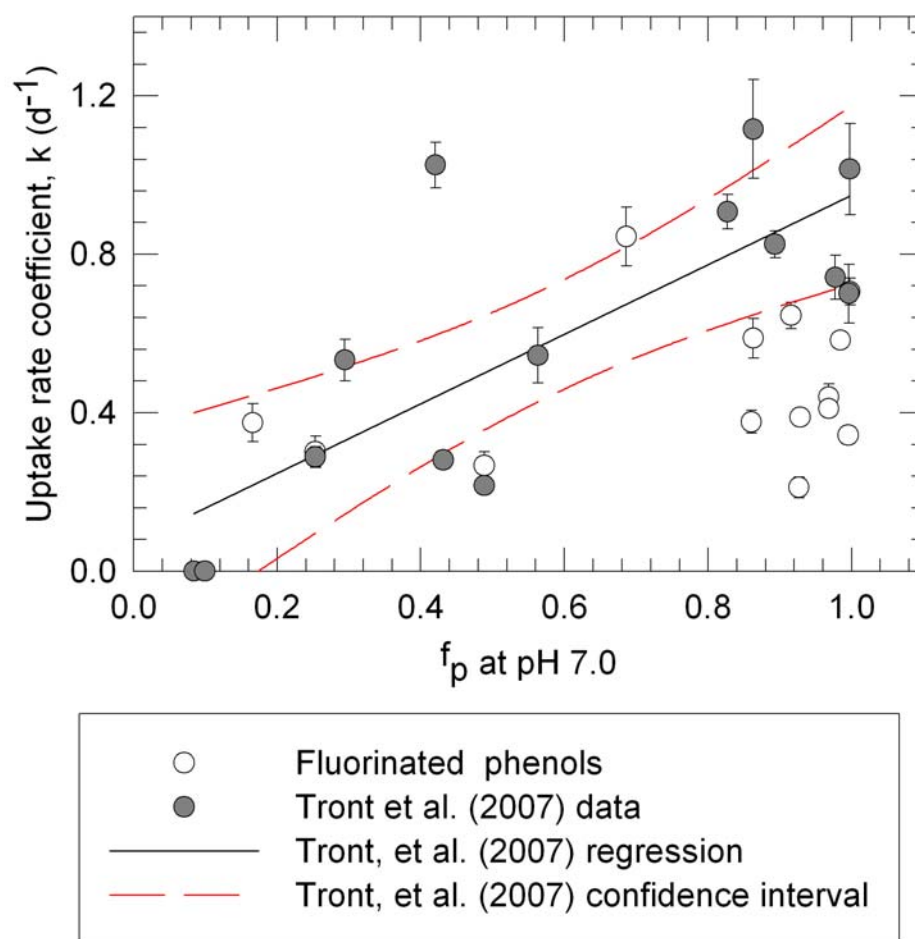


Figure 3.4. Uptake rate coefficients versus fraction of halogenated phenol protonated at a pH representative of intercellular pH (pH 7.0). Error bars represent standard error based on triplicate reactors.

coefficients and fraction of fluorinated phenol protonated at pH 7.0 could be hidden by uncertainties and error associated with estimated and experimental pK_a values.

Nonetheless, fluorinated phenol results were inconsistent with the previous hypothesis that fraction of organic chemical protonated at cytosolic pH is correlated with uptake rate coefficients. Consequently, while the electronegativity of the substituents may play a role in destabilizing the ring to allow faster glucosylation (as observed for monotrifluoromethylphenols vs. monofluorophenols), pK_a could not be used to predict uptake rates when external partitioning was not a factor. It is important to note that pK_a was an important parameter affecting partitioning of organic pollutants into plant cells when the external medium pH was near or less than the pK_a (Tront and Saunders 2006).

Relationships between physicochemical properties related to partitioning, such as hydrophobicity (K_{OW}), and uptake rate coefficients were also investigated. Partitioning parameters were not related to uptake rate coefficients and attempts to correlate K_{OW} with uptake rate coefficients using linear, exponential, Gaussian, and polynomial relationships did not yield statistically valid relationships. These results were consistent with a previous study on uptake of halogenated phenols by *L. minor* (Tront et al. In review), in which the lack of relationship between uptake rate coefficients and $\log K_{OW}$ was thoroughly investigated and discussed. However, uptake rate coefficients were related to position and type of fluorinated substituent. Variation in uptake rate coefficients suggested that enzymatic processing predominated partitioning as the limiting step in uptake of fluorinated and trifluoromethylated phenols. Furthermore, observations on relationships between uptake rate coefficients for subsets of fluorinated and trifluoromethylated phenols indicates that multiple isoenzymes may be responsible for enzymatic processing of these similarly structured organic pollutants in *L. minor*.

Effect of nitro- substituents on fluorinated phenol uptake

Uptake of TFM, a lampricide used in the Great Lakes, was studied to investigate the

impact of *L. minor* on aqueous depletion of aquatic pesticides. Aqueous removal of approximately 14 μM TFM in 50 h was not statistically significant (Figure 3.5), even though oxygen production rates indicated that *L. minor* was not inhibited by TFM. As 3-TFMeP and 2,4,6-trinitrotoluene (TNT) were rapidly removed (Figure 3.5), introduction of a charged nitro-group to the phenolic-ring did not prevent partitioning of TFM into plant tissue. It is important to note that, in contrast to predicted plant metabolism pathways for fluorinated phenols, TNT is phototransformed by aquatic plants through reduction of nitro groups (Hughes et al. 1997; Bhadra et al. 1999); consequently, experiments with TNT only addressed the hypothesis that charged nitro- groups prevented abiotic partitioning into *L. minor* and did not address potential differences in metabolic processing rates of TNT, TFM, and 3-TFMeP. Therefore, inability of *L. minor*

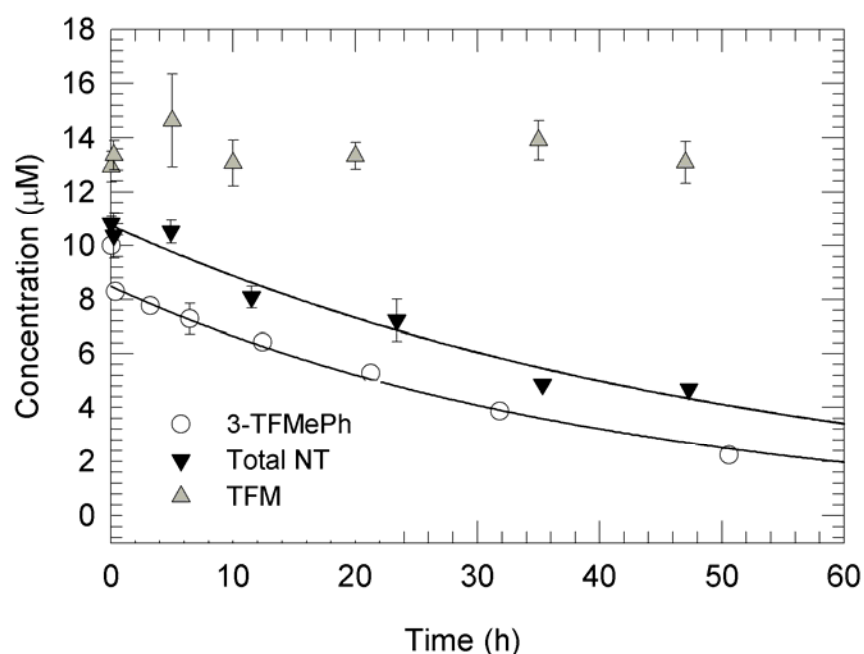


Figure 3.5. Aqueous concentration of 3-trifluoromethylphenol (3-TFMeP), total nitrotoluenes (Total NT), and 3-trifluoromethyl-4-nitrophenol (TFM) with time. Total NT concentration represents sum of parent 2,4,6-trinitrotoluene (TNT) and amino-dinitrotoluenes. Lines represent 2-parameter, first-order exponential decay relationships. Error bar represent standard deviation of triplicate reactors.

to uptake TFM was attributed to an absence of intrinsic metabolic pathways capable of transforming or conjugating TFM and was not associated with partitioning processes.

Summary

Understanding the environmental fate of organic pollutants is essential to protecting human health, endangered species, and future food and water resources. Additionally, characterization of chemical uptake rates in *L. minor* systems is essential for the future design of phytotechnologies intended for removal of fluorinated organic pollutants from water resources. This study advanced understanding of attenuation of fluorinated organic pollutants by *L. minor* and examined uptake processes that occur in both natural ecosystems and engineered wetlands designed for nutrient removal. Uptake of fluorophenols and trifluoromethylphenols by *L. minor* was a rapid process, indicating great potential for aquatic plants to remediate fluorinated organic pollutants in both engineered and natural systems. Uptake was dependent on fluorinated substituent type and position, but was independent on partitioning related properties, such as K_{OW} and pK_a . Results indicated that enzymatic processing of fluorinated phenols by plants is an important mechanism of uptake and the presence of multiple isoenzymes capable of conjugating structurally similar organic pollutants was suggested. Additionally, uptake of TNT by *L. minor* indicated that the nitro-substituent of TFM did not prevent uptake – emphasizing again the importance of enzymatic processing in uptake of organic pollutants by aquatic plants.

CHAPTER 4: CONCENTRATION EFFECTS ON UPTAKE AND PLANT ACTIVITY IN *LEMNA MINOR* SYSTEMS

Introduction

Contamination of surface waters and ground waters by halogenated pesticides, pharmaceuticals, and personal care products (Kolpin et al. 2002; Taniyasu et al. 2003; So et al. 2004; Boulanger et al. 2005) prompts concerns about the environmental fate and persistence of these anthropogenic chemicals. Fluorinated organic chemicals represent a growing portion of these compounds. Because of their low chemical reactivity, fluorinated organic chemicals persist in the environment and bioaccumulate in human, fish and bird tissues (Taniyasu et al. 2003). Consequently, understanding the environmental fate of fluorinated organic pollutants is essential to protecting human health, endangered species, and future food and water resources.

Despite the crucial role aquatic plants play in removing many organic pollutants from surface water supplies (Bhadra et al. 1999; Crum et al. 1999; Gao et al. 2000; McCutcheon and Schnoor 2003), active plant-driven accumulation and metabolism of organic pollutants is often neglected in consideration of environmental fate. However, plants may serve as reservoirs for fluorinated organic pollutants and as conduits for introducing fluorinated organic pollutants into animal and human food supplies. Through sorption, diffusion, and partitioning, aqueous-phase pollutants accumulate inside plant cells (Day and Saunders 2004). Continued uptake depends on plant metabolism of these internal organic pollutants – a serial chain of biochemical reactions that can include transformation of parent pollutants, conjugation of metabolites with macromolecules, and incorporation of conjugated products into cell walls and vacuoles (McCutcheon and Schnoor 2003). This packaging of pollutants into plant biomass eliminates phytotoxicity associated with the parent compound and may also facilitate ultimate mineralization

during anaerobic decay of dead plant biomass (Tront et al. 2006).

Lemna minor, a species of duckweed, is a key aquatic plant in natural and engineered water systems. *Lemnaceae* are frequently used as model plants to study toxicity of pollutants and toxicity assessments using *Lemna* species have been standardized by multiple organizations, including American Public Health Association, American Water Works Association, and Water Environment Federation (APHA et al. 1998b) and American Society for Testing and Materials (ASTM 1997). Toxicity tests using *Lemna* species utilize a variety of experimental procedures and analytical techniques. Generally, these standardized tests use a low number (e.g., 10 – 20) of plant fronds in test vessels at surface coverages that are extremely low, especially when compared to surface coverages representative of natural waters, wetlands, or oxidation pond systems. Instead, a maximum of 12 fronds (APHA et al. 1998b) or 16 fronds (ASTM 1997) are exposed to aqueous pollutants for 7 d. After exposure, frond health or growth is assessed by one or multiple endpoints, including frond number, dry biomass, and root length. Biochemical end-point assessments include oxygen production (Tront and Saunders 2006), photosynthetic oxygen evolution (Huber et al. 1982; Geoffroy et al. 2004), and chlorophyll fluorescence (Huber et al. 1982; Hulsen et al. 2002; Brain et al. 2004a; Geoffroy et al. 2004).

Lemnaceae species are valuable in researching the fate of organic pollutants in aquatic systems. At full surface coverage, *L. minor* quickly processes halogenated aromatic compounds; the aqueous-phase half-life for removal of 8 μ M 2,4,5-trichlorophenol (2,4,5-TCP) by *L. minor* (10g fresh mass/L) is 16 to 22 h (Tront et al. 2007). Primary metabolism of 2,4-dichlorophenol (2,4-DCP) and 2,4,5-TCP proceeds primarily through glycosidation in *L. minor*, yielding chlorinated malonyl and apiose conjugates that are incorporated into vacuoles and cell walls (Day and Saunders 2004; Pascal-Lorber et al. 2004). Assimilation of 2,4-DCP following conjugation with biomolecules by *L. minor* concurs with published data. Pentachlorophenol, 2,4,5-

trichlorophenol, 2,4-dichlorophenoxyacetic acid, and phenol are all reported to undergo glycosylation, particularly glucosylation, in plants (Frear et al. 1983a; Casterline Jr. et al. 1985; Barber et al. 1995; Sharma et al. 1997; Laurent et al. 2000). It is hypothesized that similar transformation pathways occur for most halogenated phenols, including fluorinated and trifluoromethylated phenols; however, this pathway has only been demonstrated for acifluorfen, where intermediate 2-chloro-4-trifluoromethylphenol was glucosylated (Frear et al. 1983a). Uptake rate of 2,4-DCP, including conjugation and sequestration, has previously been demonstrated to depend on plant activity, as assessed by 24-h oxygen production by *L. minor* (Tront and Saunders 2006). These results were for non-toxic levels of 2,4-DCP.

This study focused on determining the relationships between uptake rates and concentrations for two similar fluorinated phenols, 3-fluorophenol (3-FP) and 3-trifluoromethylphenol (3-TFMeP). To distinguish concentrations effects on uptake from inhibition of *L. minor*, inhibitory effects of 3-FP and 3-TFMeP were assessed using oxygen production rate assessment. Additional objectives included enhancing the oxygen production assessment (Tront and Saunders 2006) to increase sensitivity and reliability of response while decreasing total assessment time.

Materials and Methods

Materials and supplies

3-Fluorophenol (3-FP) and 3-trifluoromethylphenol (3-TFMeP) were used to examine uptake of fluorinated organics by *L. minor*. Fluorinated phenols were obtained from Alfa Aesar with 98% purity. *L. minor* was collected from a wastewater reclamation wetland operated by the Clayton County Water Authority. Stock *L. minor* was photoautotrophically maintained under simulated natural conditions in laboratory tanks with nutrients supplied from either potting soil or wetland sediments. *L. minor* was

cultured under an 18-h photoperiod in a growth chamber at 22°C. *L. minor* was thoroughly rinsed with DI water and quantified with fresh mass prior to experiments, where fresh mass was determined after removal of excess water through a brief air-drying period and blotting.

Oxygen production rate (OP) assessment

L. minor activity was assessed using oxygen production analysis prior to and after uptake and toxicity assessments. For each replicate analysis, two sealed reactors were incubated at 22 °C, with one exposed to the light and one maintained in the dark. Reactors consisted of 160-mL serum bottles with 60-mL medium and specified fresh mass of *L. minor*. For optimization studies, Standard Methods medium (SM) for duckweed toxicity assessments (APHA et al. 1998b) was modified with 0.06-50.06 mM phosphate buffer (pH 7) and 1.8-11.8 mM additional carbonate. Incubation times of 2-14 h and 0.2-1.0 g fresh mass *L. minor* were assessed. At the end of incubation, change in total headspace volume at atmospheric pressure was measured with manometers designed to prevent carbon-dioxide and oxygen scavenging (APHA et al. 1998a). Headspace oxygen was analyzed by GC-TCD with separation by molecular sieve and helium as the carrier gas. The μmol of oxygen produced by *L. minor* was calculated by subtracting oxygen in the dark reactor from the oxygen in the light reactor, where the dark reactor accounted for oxygen consumed by respiratory processes. Consequently, oxygen production assessments measured total oxygen produced by photosynthesis. Oxygen production rate (OP) was calculated by normalizing oxygen produced with time and initial fresh mass of *L. minor* and was expressed in units of $\mu\text{mol O}_2/\text{h-g } L. \text{ minor}$.

Natural variability of *L. minor* activity in the Clayton County Water Authority (CCWA) constructed treatment wetlands from June 2004 to July 2005 was 4.5 ± 0.3 to $30.2 \pm 2.5 \mu\text{mol/h-g}$ (Figure 4.1). While frequency of dense *L. minor* growth was observed to decrease during fall and winter months at CCWA wetlands, a seasonal trend

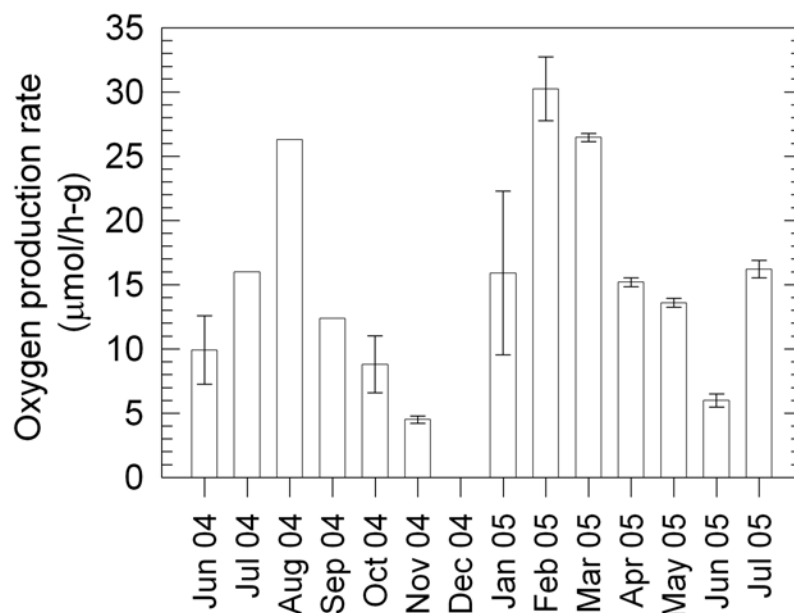


Figure 4.1. Oxygen production rates for *L. minor* communities collected from Clayton County Water Authority treatment wetlands during June 2004 to July 2005. Error bars, when present, represent standard deviations of a minimum of triplicate reactors.

was not apparent in *L. minor* OP. It is important to note that water temperature fluctuations due to seasonal variations were minimized due to the relatively constant influent water temperature into the wetland. Many potential contributing factors were observed in months with low OP (e.g., <10 μmol/h-g), including increased shading in some wetland cells, lower temperatures, and insect and fungal infestation.

Uptake and toxicity assessments

Uptake of 3-FP and 3-TFMeP was measured in 160-mL open, batch reactors containing 60-mL modified SM and 0.5 g fresh mass *L. minor*. Phosphate buffer (10 mM P) was added to SM to minimize pH changes, yielding a final phosphate buffer concentration of 10.06 mM P or 312 mg/L P. Fresh mass of 0.5 g *L. minor* was added to 160-mL reactors, as this quantity provided full surface coverage. Stock 3-FP or 3-TFMeP was added to achieve desired concentrations ranging from 10 μM to 1750 μM. Aqueous concentration of 3-FP or 3-TFMeP was regularly quantified with HPLC-

UV/Vis-MSD for duration of uptake measurements and toxicity exposure. Contaminant exposure was conducted under continuous light at 22°C for 24 h or 50 h.

Uptake rates for 3-FP and 3-TFMeP were determined from aqueous concentration data, given that there were no volatilization losses. Aqueous concentrations over time were normalized to initial concentration measurements (i.e., C_I = concentration determined for sample taken at t_I =10 min following addition of plants to the medium). Sorption studies have indicated that rapid equilibration occurs in the first 10 min of exposure to inactivated *L. minor* (Tront and Saunders 2006). Therefore, normalization to C_I samples accounted for equilibration and for water additions made with addition of plants to reactors. Uptake rate constants were established by fitting normalized concentration data with 2-parameter, first-order exponential decay relationships with Sigma Plot 2000.

Toxicity of 3-FP and 3-TFMeP to *L. minor* was assessed via the OP assessment under conditions similar to uptake studies. *L. minor* (0.5 g fresh mass) was exposed to concentrations of 3-FP or 3-TFMeP ranging from 0-1750 μ M in 60-mL modified SM in open, batch reactors. After an exposure of 24 h, the medium was replaced with fresh, no-contaminant medium. The reactors were then sealed and divided into triplicate “light” and “dark” reactors. Reactors were kept at 22°C in either continuous light or dark conditions for ~6 h. Total μ mol of oxygen gas in the reactor headspace was determined from measurements of %O₂ (GC-TCD) and change in total headspace volume (manometer). OP in units of μ mol O₂/h-g *L. minor* was calculated by subtracting oxygen consumed from respiration in the dark reactor from net oxygen produced from photosynthesis and respiration in the light reactor and dividing by total incubation time (t~6 h).

Results and Discussion

Oxygen production rate (OP) assessment

Oxygen production rate (OP) by *L. minor* in closed systems for 24 h was previously used to determine inhibition of *L. minor* during uptake studies (Tront and Saunders 2006; Tront et al. 2007). The responses of OP to time, buffer concentration, carbonate concentration, and mass *L. minor* were explored in this study to improve sensitivity and reliability of the assessment.

The dependence of μmol oxygen produced over 2-12 h on concentration of carbonate was investigated at 1.8, 5.8, 6.8, and 11.8 mM total carbonate in SM with 0.5 g fresh mass *L. minor*. Systems were buffered with 10.06 mM phosphate buffer (pH 7) and negligible pH changes were observed. The quantity of oxygen produced increased linearly with time, and slopes of oxygen produced (μmol) versus time (h) were statistically similar for all concentrations (Figure 4.2). Statistical similarity of the rates of oxygen production (per unit time), as determined by analysis of μmol oxygen produced over time, indicated independence of OP on carbonate concentration. Consequently, it was concluded that carbon was not limiting for OP from 2 – 12 h and SM contained sufficient carbon for use in the OP assessment.

Oxygen produced by *L. minor* was monitored over 2 – 14 h with phosphate buffer concentrations of 0.06, 5.06, 10.06, 20.06, and 50.06 mM P (initial and buffer pH=7). Oxygen produced in systems at 5.06 – 20.06 mM P were greater than in 0.06 mM P systems, as presented in Figure 4.3. Oxygen produced was linear with time ($r^2 > 0.93$, $p < 0.0001$) for systems with ≥ 5.06 mM P. Analysis of slopes of μmol of oxygen produced versus time indicated statistical similarity at phosphate buffer concentrations of 10.06 – 50.06 mM P. Results indicate necessary modifications to SM medium for this application, and do not necessarily imply an insufficiency in SM medium when used in

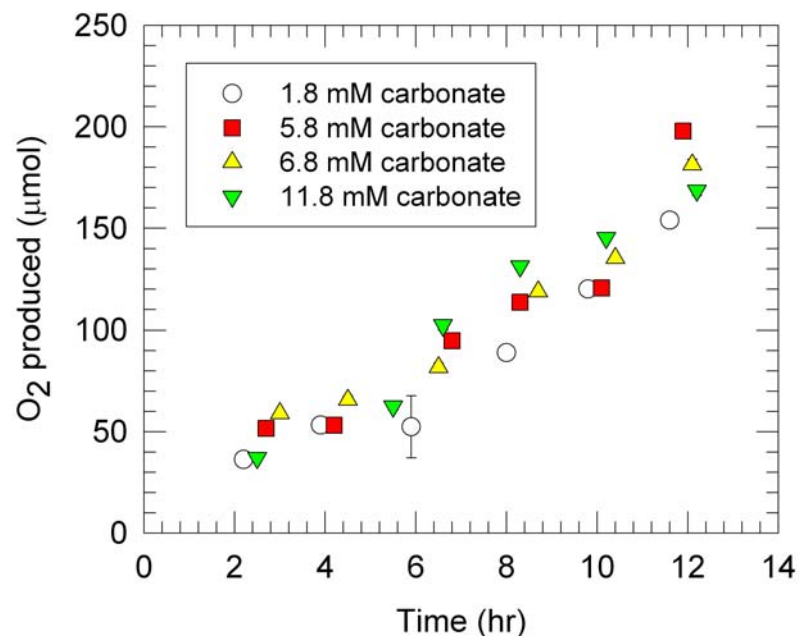


Figure 4.2. Oxygen produced with time for carbonate concentrations of 1.8 mM, 5.8 mM, 6.8 mM, and 11.8 mM. Error bars (not always visible) represent standard error of analysis (n=4)

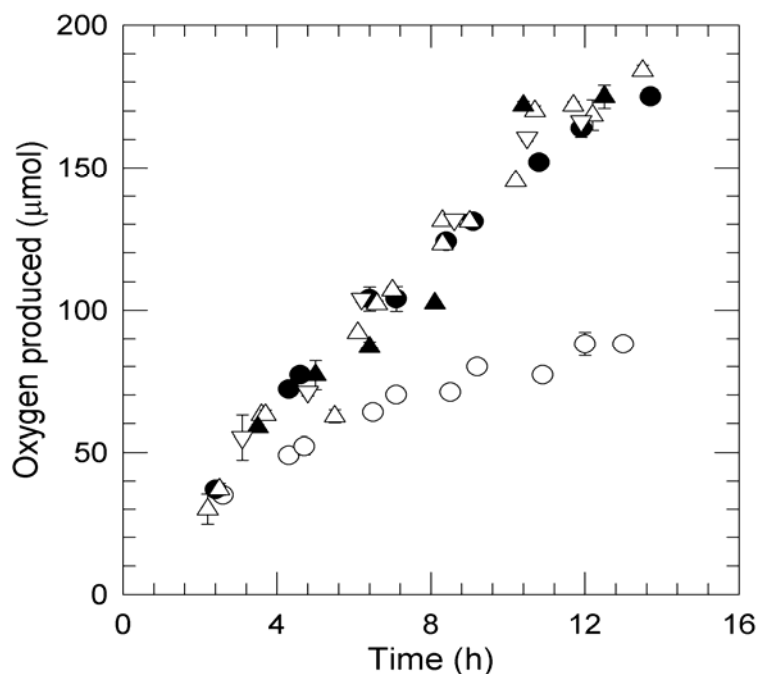


Figure 4.3. Effect of controlling pH variations with increasing phosphate buffer concentrations on O₂-produced by 0.5 g fresh mass *L. minor*. Error bars represent standard error of analysis (n=4). ○ = 0.06 mM P; ● = 5.06 mM P; △ = 10.06 mM P; ▲ = 20.06 mM P; ▽ = 50.06 mM P.

conventional toxicity assessments utilizing open reactors and 12 – 16 fronds as carbon fixation is likely substantially less in conventional toxicity assessments than in OP assessment developed in this study.

Final pH at t=14 h for SM with 0.06, 5.06 and 10.06 mM phosphate buffer were 7.0 in the closed reactors systems. Negligible pH change (≤ 0.2 units) was observed at higher buffer concentrations. OP decreased linearly with increasing final medium pH of closed light reactors ($r^2=0.94$, $p<0.01$). As the ability of the plants to regulate vacuolar and cytosolic pH is hindered at $\text{pH}>7.5$ (Gout et al. 1992), it was considered plausible that OP at external $\text{pH}>7.5$ was hindered by increasing intracellular pH and consequent effects on enzymatic catalysis and proton gradients involved in photosynthesis. An additional explanation for the decrease in OP with increasing medium pH was a decrease in CO_2 and HCO_3^- -- the two available forms of carbon for aquatic plants (Falkowski and Raven 1997). Subsequent studies were conducted at 10.06 mM P to minimize pH changes and assure adequate nutrient levels.

OP was constant at $26.76 \pm 1.08 \mu\text{mol/h-g}$ over 2 – 14 h when medium was buffered with 10.06 mM phosphate (Figure 4.4). SM contained sufficient carbon (i.e., 1.8 mM C) for incubation times less than 12 h and additional carbonate was considered unnecessary. The linear trend of O_2 -produced (μmol) with time for 0-12 h justified shortened incubation times for subsequent studies. An incubation time of 6 h was selected for toxicity studies to allow for entire assessment, including set-up, incubation, and analysis, to be completed in an 8-h period.

Plant density effects on oxygen production rate (OP)

The effect of plant density and surface coverage on OP by *L. minor* was examined for *L. minor* masses of 0.2-1.0 g per 60 mL of SM with 10.06 mM phosphate buffer (initial pH 7). Qualitative observation indicated that 100% surface coverage of *L. minor* for the reactors occurred with masses of approximately 0.43 g and higher.

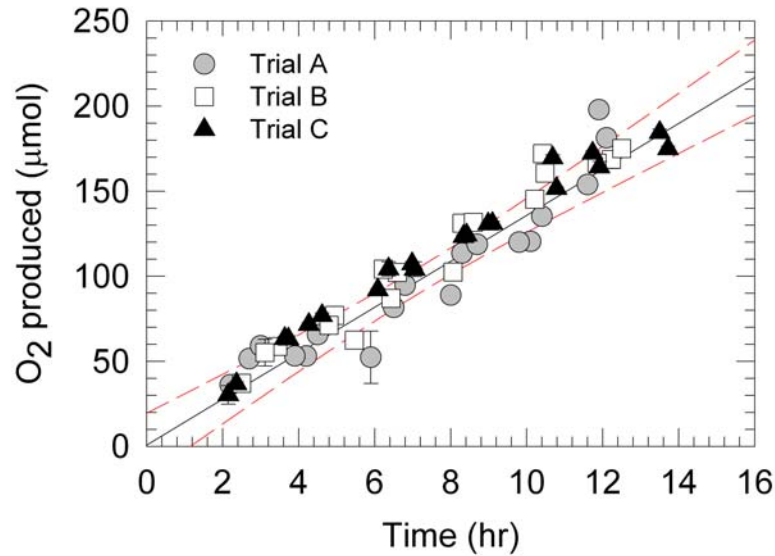


Figure 4.4. Constant oxygen production ($\mu\text{mol/h}$) for 0.5 g *L. minor* for 2-14 h in three separate trials over 15 days. Points represent average of duplicate analysis and error bars represent 95% confidence intervals. Solid linear-regression line is statistically significant (i.e., $r^2=0.96$, $p<0.0001$) with slope of $13.38\pm0.54 \mu\text{mol/h}$ or $26.76\pm1.08 \mu\text{mol/h-g}$, when normalized to mass *L. minor*.

As fresh mass increased past 0.43 g, crowding and multiple layers or matting of *L. minor* occurred, similar to conditions frequently observed in natural environments. OP, as affected by fresh mass, are presented in Figure 4.5 for fresh masses of 0.2 to 1.0 g (i.e., $0.10 - 0.51 \text{ kg/m}^2$). Maximum OP observed was $25.7\pm0.6 \mu\text{mol/h-g}$ at 0.2 g *L. minor*, the lowest fresh mass investigated. OP decreased hyperbolically with increasing mass of *L. minor* to a minimum of $9.25\pm0.49 \mu\text{mol/h-g}$ at 1.0 g *L. minor*, indicating that surface coverage was a key variable in all systems examined. As OP is integrally related to plant activity and rates of uptake of contaminants, these results have considerable significance in natural environments and engineered wetland systems. Experimental systems were not investigated at lower mass values, or at multiple-frond levels discussed above.

Dependence of OP on plant density was consistent with observations of decreasing growth rate of *L. minor* with increasing plant surface coverage (Driever et al. 2005).

Driever et al. (2005) observed decreased biomass production with increased plant

density, as characterized by:

$$\left(\frac{dB}{dt}\right)\left(\frac{1}{B}\right) = r \left(\frac{T - T_{\min}}{T_{\text{opt}} - T_{\min}}\right) \left(\frac{N}{N + h_N}\right) \left(\frac{P}{P + h_P}\right) \left(\frac{h_B}{B + h_B}\right) - l \quad \text{Equation 4.1}$$

hyperbolically with increasing mass of *L. minor* to a minimum of $\sim 10 \mu\text{mol/h-g}$ at 1.0 g where B was biomass surface coverage (g DW/m^2), r was maximum growth rate (d^{-1}), l was loss (d^{-1}). Biomass production was dependent on air temperature (T), surface coverage (B), nitrogen concentration (N) and phosphorus concentration (P). The parameters h_N , h_P , and h_B represented half-saturation values for nitrogen, phosphorus, and surface coverage limitations on growth and had values of 0.04 mg/L N (Luond 1980), 0.05 mg/L P (Luond 1980), and 26 g dry mass/ m^2 (Driever et al. 2005), respectively. The minimum and optimal temperatures (T_{\min} , T_{opt}) were 5°C and 26°C, respectively (Landolt 1986; Landolt and Kandeler 1987). Determined values for r and l were 0.41 d^{-1} and 0.05 d^{-1} , respectively.

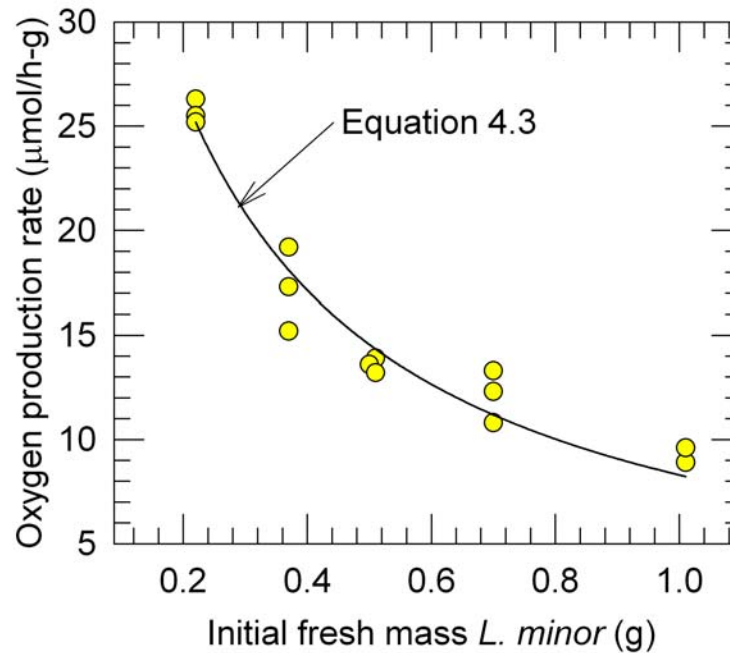


Figure 4.5. OP by *L. minor* for increased initial plant mass in 19.6- cm^2 surface-area reactor. Solid line represents model results from Equation 4.3. The half-saturation value ($h_{M,OP}$) for initial fresh mass *L. minor* on OP was 0.163 ± 0.037 g.

Decrease in OP with increasing mass was compared to the concept presented by Driever et al. (2005) to examine effects of surface coverage on OP. Biomass loss rate (l) was considered negligible for the OP assessment because the OP assessment employed herein accounted for endogenous respiration and loss from death and predation was assumed negligible over 6 h. Fresh mass of *L. minor* was projected to be synonymous to plant density for the closed reactors as volume (i.e., 60 mL) and surface area (i.e., 19.6 cm²) of SM was constant for all reactors. Using OP, in units of $\mu\text{mol O}_2/\text{h-g } L. \text{ minor}$, as a representative parallel parameter for the normalized growth rate, $\left(\frac{dB}{dt}\right)\left(\frac{1}{B}\right)$ of Driever et al. (Driever et al. 2005) the relationship in Equation 2 was proposed for OP:

$$OP \propto r \left(\frac{T - T_{\min}}{T_{\text{opt}} - T_{\min}} \right) \left(\frac{N}{N + h_N} \right) \left(\frac{P}{P + h_P} \right) \left(\frac{h_B}{B + h_B} \right) \quad \text{Equation 4.2}$$

In the systems examined, temperature was constant at 22°C and N and P levels were well above any limitations, making their perspective terms approach unity. Therefore, mathematically, the data in Figure 3 was examined using the resulting relationship:

$$OP = a \cdot \frac{h_{M,OP}}{M + h_{M,OP}} \quad \text{Equation 4.3}$$

where a is a proportionality parameter, $h_{M,OP}$ is the “half saturation” value for surface coverage, and M is initial fresh mass of *L. minor*. The resulting equation accurately described the observed relationship between OP and mass of *L. minor* (i.e., $r^2 > 0.97$, $p < 0.0001$) and is represented as the model line in Figure 4.5.

In examination of this relationship (Figure 4.5 and Equation 4.2), the proportionality parameter a has units of OP and a value of $59.5 \pm 9.1 \mu\text{mol/h-g}$. It is important to note that this value is outside the experimental investigation and is not presumed to describe processes in these experimental systems or associated biochemical processes. The relationship does however provide an effective means for examination of the experimental reactors and an integrated outcome of relevant physicochemical and

biochemical processes. The $h_{M,OP}$ value of 0.163 ± 0.037 g indicated that substantial crowding occurred at all experimental masses (0.2 – 1.0 g), including masses with less than full surface coverage (0.2 – 0.4 g).

Dependence of OP by *L. minor* on plant density has implications for use of *L. minor* in contaminant uptake and toxicity studies. According to work by Tront and Saunders (2006), decreased OP by *L. minor* at high plant densities would indicate a corresponding decrease in contaminant uptake rates. Additionally, the developed relationship emphasizes the dynamic nature of growth of *Lemnaceae* and the complexity of comparing contaminant uptake and toxicity presented in literature where plant densities vary from limited fronds per reactor to densities similar to those used herein (e.g., 25.5 mg fresh mass/cm²). It is important to note that uptake rates, as presented in this study, are pseudo-first order rate coefficients that are dependent on many unknown and known parameters, including plant activity and inhibition (Tront and Saunders 2006) and plant mass (McCutcheon and Schnoor 2003). Therefore, dependence of OP on plant mass suggests that plant mass can directly and indirectly affect uptake of organic pollutants, via effects of uptake and plant activity, respectively.

Fresh mass of 0.5 g *L. minor* was used for the subsequent OP and uptake assessments, as this value approximated naturally observed surface coverage and minimized light partitioning of the underlying water column in experimental systems.

Plant activity of *L. minor* exposed to 3-TFMeP and 3-FP

Potential toxicity due to uptake of 3-FP and 3-TFMeP was analyzed via OP. Response of OP by *L. minor* to 3-TFMeP and 3-FP was measured in two separate trials to demonstrate that reproducible results could be obtained at shortened exposure periods of 24-h and 48-h for Trials A and B, respectively. The control (i.e., non-exposed) OP values were 12.9 ± 0.2 and 15.2 ± 0.3 $\mu\text{mol/h-g}$ respectively for trials A and B. This natural variability in OP is attributable to differences in collection date, “age” of *L. minor*

collected, genotype, and length of laboratory culture prior to experimentation. Similar responses in plant activity were observed for both trials when exposed OP was normalized to control OP. *L. minor* OP was substantially impaired by increasing concentration of 3-TFMeP but was not impacted by increasing concentration of 3-FP, as presented in Figure 4.4. Normalized OP was significantly impacted at $>700 \mu\text{M}$ 3-TFMeP. 3-parameter sigmoid relationships have been used previously to describe toxicities of organic pollutants to *Lemna gibba* (Brain et al. 2004a) and decrease in normalized OP with increasing concentration of 3-TFMeP was described by a 3-parameter sigmoid relationship (Systat Software Inc. 2000) with an EC_{50} of $675 \pm 37 \mu\text{M}$. A general trend of non-response was observed for concentrations of $10 - 1750 \mu\text{M}$ 3-FP. In sum, the response of OP by *L. minor* to 3-TFMeP and 3-FP was reproducible and accounted for variability associated with natural, biological samples.

Visual observations of *L. minor* under full surface-coverage conditions (i.e., 0.5 g per 60 mL) provided no indication of plant activity or toxicity. *L. minor* exposed to toxic concentrations of 3-TFMeP under these full surface-coverage conditions was visually similar to non-exposed *L. minor*. At the most toxic conditions in this experiment (i.e., $>1250 \mu\text{M}$), the only repeatable observation was a slight yellow tint to the medium at exposure lengths greater than 40 h. It was hypothesized that this yellow tint resulted from leaching of phenolic compounds upon frond stress or from release of transformed phenols by plants. However, *L. minor* fronds under full-surface coverage conditions appeared healthy when judged by size, quantity, and color – even at the highest concentrations tested. These observations emphasized the need for sensitive assessments of frond health and activity under natural growth conditions, such as OP by *L. minor*.

To compare the sensitivity of OP by *L. minor* under full surface coverage conditions to standardized toxicity assessments utilizing a limited number of fronds, chlorosis of single fronds was measured for 3-TFMeP. Chlorosis measurements utilized plant density of 12 fronds per 78.5 cm^2 . At 7 d, partial chlorosis ($\sim 50\%$) of fronds was

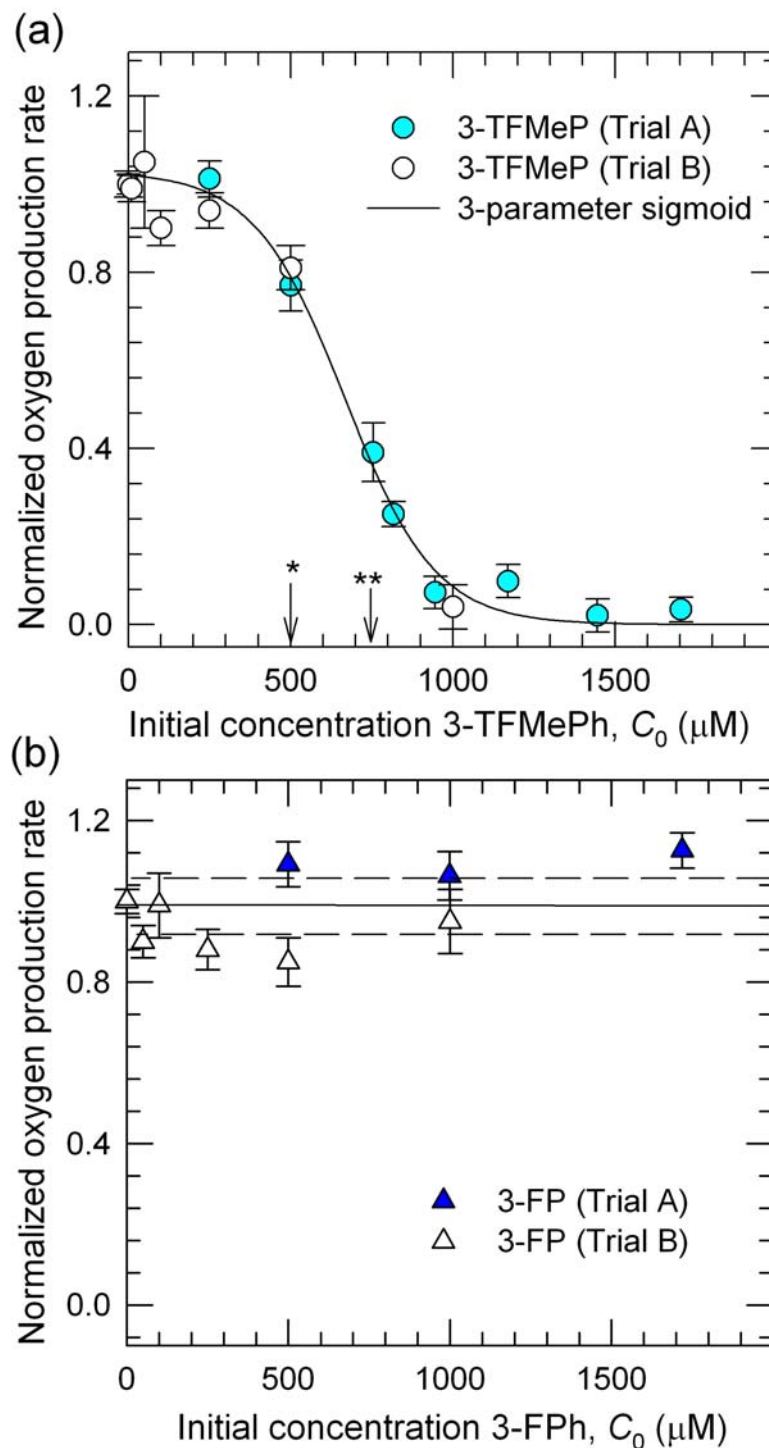


Figure 4.4. Response of normalized OP to increasing concentrations of 3-TFMeP (a) and 3-FP (b). OP is normalized to control (i.e., non-exposed) *L. minor*. Control OP was 12.9 ± 0.2 and 15.2 ± 0.3 $\mu\text{mol/h-g}$ for Trial A and Trial B, respectively. Points represent mean of four replicates and error bars represent 95% confidence intervals. Mean control OP for 10 – 1750 μM 3-FP was 0.983 ± 0.063 $\mu\text{mol/hr-g}$. * and ** indicate partial and complete chlorosis, respectively, measured in 12-frond exposure trial (see text).

observed at 500 μM and complete chlorosis was observed at 1000 μM , concentrations that corresponded with $\sim 20\%$ and $\sim 60\%$ reduction in normalized OP (Figure 4.4a). Consequently, OP by *L. minor* was a reasonable assessment for toxicity of 3-TFMeP. Additionally, with an exposure period of 24 h to 48 h, an incubation time of 6 h, and analysis time of 5 min, OP by *L. minor* was well suited to assess plant activity in conjunction with short-term (i.e., < 50 h) uptake studies. Analysis by GC-TCD was easily quantified as compared with labor-intensive endpoints (e.g., counting fronds, analyzing % chlorosis). As such, OP by *L. minor* provided a viable, rapid alternative for assessing toxicity of pollutants to *L. minor* in conjunction with uptake assessments.

Removal of 3-TFMeP and 3-FP

From the initial measurement at $t_1=10$ min to the final measurement at $t=50$ h, removal of fluorinated phenols from aqueous phase by *L. minor* generally followed exponential decay relationships; examples of which are shown in Figure 4.5. Decreases in normalized concentrations (i.e., C/C_I) were well-represented statistically (i.e., $p \leq 0.001$, $r^2 > 0.80$) by 2-parameter exponential decay relationships. An uptake rate constant of zero indicated a constant plateau of aqueous concentration and failure of fit to pass statistical validity (i.e., $p > 0.001$).

Uptake rate constants (k) decreased with increasing concentration of 3-FP or 3-TFMeP (Figure 4.6a). The trend between uptake rate constants and concentration was well represented by a modified, 3-parameter exponential decay. Decrease in plant activity due to toxicity did not account for the decrease in uptake rate constants with increasing concentration for concentrations of 10 to 250 μM 3-TFMeP or 10 to 100 μM 3-FP. For 3-TFMeP, OP by *L. minor* at 250 μM was statistically similar to the control OP in both trials, indicating that inhibition of *L. minor* was not responsible for the decrease in k -values over this concentration range. Concentration-driven effects on uptake rates were also independent of concentration effects on plant activity and

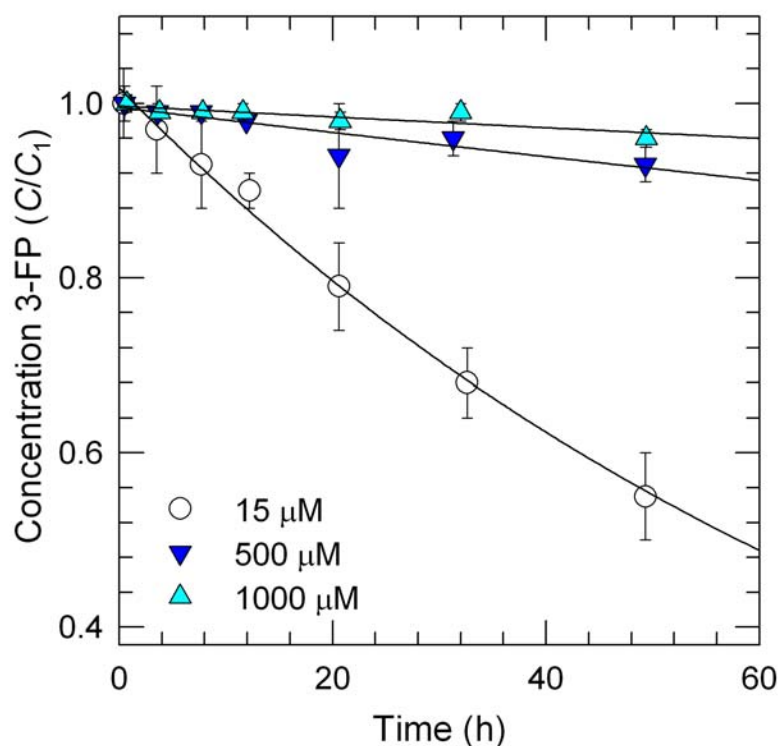


Figure 4.5. Uptake of 3-FP by *L. minor* at 22°C under continuous light. Concentration is normalized to initial concentration at $t=10$ min. Points represent means of six replicates and error bars represent 95% confidence intervals.

inhibition, as assessed via OP, for 3-FP, where decrease in uptake rate coefficients occurred when OP was constant (i.e., 10 – 1000 μM 3-FP). Additionally, similar decreases in uptake rate constants were observed for both 3-TFMeP and 3-FP, indicating that decreasing uptake rates were not pollutant specific. Potential explanations for limited uptake at concentrations greater than 100 μM include saturation of transformation enzymes, saturation of partitioning sites, and enzyme inhibition. At concentrations >250 μM 3-TFMeP, concentration effects on plant activity are also likely contributing to decrease in uptake rate coefficients and the trend between uptake rate coefficients and concentration probably reflects a combination of concentration and activity effects. Comparison of concentrations trends for 3-TFMeP and 3-FP indicates that adverse effects of inhibited plant activity on uptake rate coefficients were minor when compared to

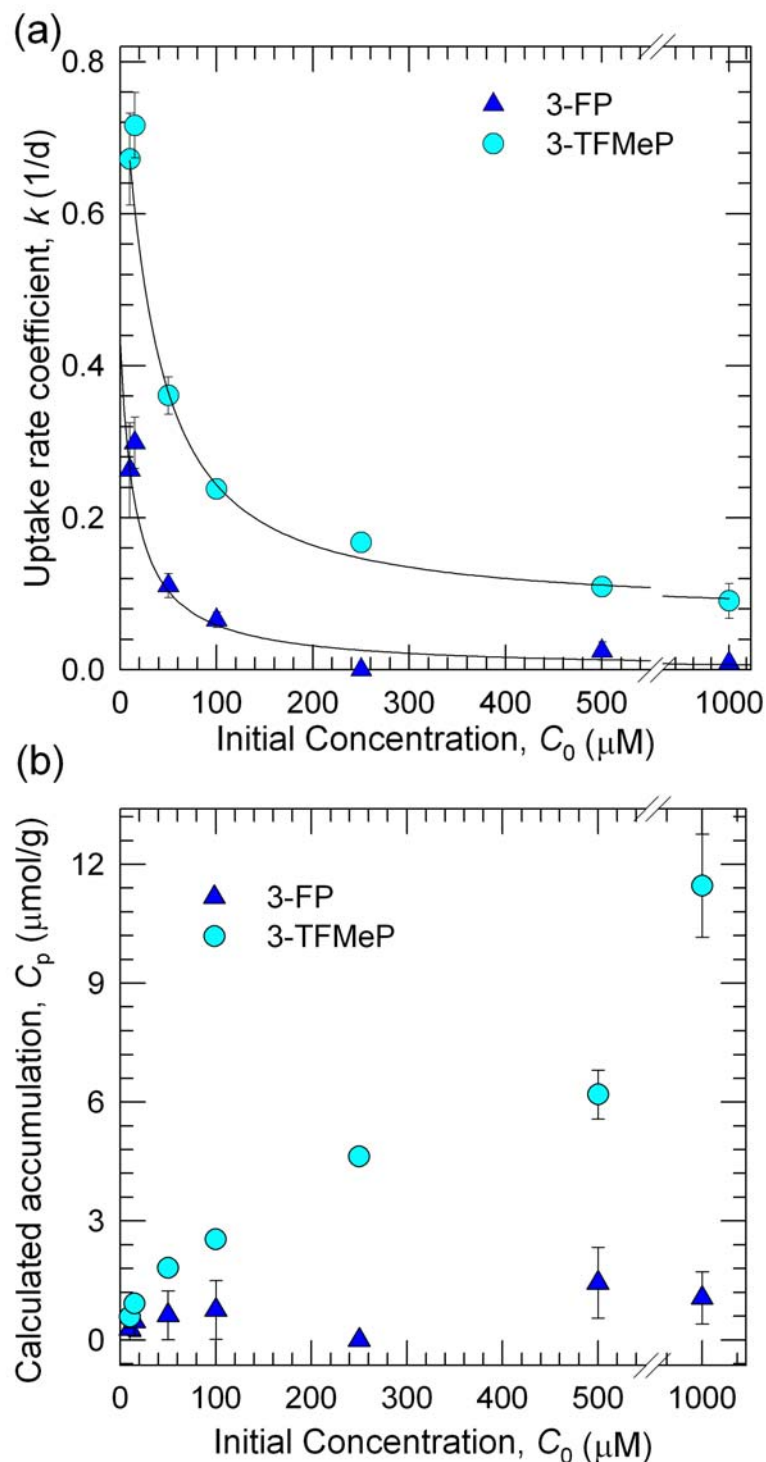


Figure 4.6. a.) Change in pseudo-first-order uptake rate constants with concentration of 3-FP or 3-TFMeP. Points represent individual uptake trials and error bars represent standard error of uptake regression. Lines represent modified single 3-parameter exponential decay fits of 3-FP and 3-TFMeP data sets. b.) Theoretical accumulation ($\mu\text{mol/g}$) of 3-FP or 3-TFMeP by *L. minor* in 24-h as calculated by Equation 4.4.

concentration-driven effects on uptake rate coefficients for 3-TFMeP. However, contribution of inhibited plant activity to concentration effects on uptake rate coefficients is expected to increase with increasing toxicity of organic pollutants; therefore, concentration effects on plant activity and uptake rate coefficients are expected to both contribute substantially to uptake rate coefficients for organic pollutants with EC50 values of <100 µM.

To further examine the impact of concentration on uptake of pollutants by *L. minor*, the theoretical accumulation of fluorinated phenols by *L. minor* in 24-h (M_{acc}^{24h}) was calculated from uptake rate constants using the following equation:

$$M_{acc}^{24h} = C_0 V / M (1 - \exp(-k \cdot 1d)) \quad \text{Equation 4.4}$$

Accumulation thus included all contaminant forms (e.g., parent, transformed, conjugated) internal to *L. minor*. In general, *L. minor* accumulated more 3-TFMeP as initial concentrations increased (Figure 4.6b). M_{acc}^{24h} of 3-FP also increased with increasing concentrations (i.e., 10 µM – 500 µM), but appeared to plateau at concentrations >500 µM. The average accumulations of 3-FP at concentrations of 500 µM and 1000 µM were statistically similarly, with values of 1.44±0.49 µmol/g and 1.06±0.15 µmol/g, respectively. This analysis indicated that maximum accumulation values may be an important parameter in removal of some pollutants by *L. minor*. However, even at elevated contaminant concentrations, *L. minor* still accumulated significant amounts of 3-FP and, through biomass growth and turn over, would still play an important role in removing 3-FP and other pollutants from natural and engineered wetland systems.

Summary

The OP assessment for *L. minor* was a reliable method to determine plant activity in conjunction with uptake studies. OP did not depend on concentration of carbonate in SM medium (1.8 – 11.8 mM) or on the incubation time (2 – 14 h). However, SM

medium required a modification of 10.06 mM phosphate buffer to prevent increasing pH during incubation, as OP was dependent of final pH. The OP assessment was also dependent on initial fresh mass of *L. minor*, with a half-saturation value ($h_{M,OP}$) of 0.163 ± 0.037 g. Consequently, crowding was a important factor in OP and may play a significant role in the uptake of pollutants by *L. minor*.

The OP assessment developed herein allowed for rapid assessment of *L. minor* activity in response to a >100-fold concentration range of 3-TFMeP and 3-FP. As compared with conventional Lemnaceae toxicity assessments using 7-d exposure periods, the OP assessment utilized a shortened exposure of 24 h and an easily quantified end-point. Reduction in OP from toxicity was consistent with independent measurements of chlorosis in a 12-frond experiment. Additionally, the OP assessment allowed for determination of toxicity of pollutants to *L. minor* grown under full surface-coverage conditions.

This study suggests that contaminant concentration dominated toxicity as a major hindrance of uptake of pollutants by *L. minor*. The pseudo-first-order rate constants, or k-values, used here-in represented pseudo parameters that included many unknown variables; however, this study demonstrated that uptake rates were not dependent upon toxicity. Additionally, despite different maximum uptake rate constants for 3-TFMeP and 3-FP, a substantial decrease in uptake rate constants at concentrations of 10-100 μ M was observed, indicating that the observed concentration trend was not an isolated phenomenon unique to these pollutants. Consequently, dependence of uptake rate constants on concentration was considered intrinsic to contaminant uptake by *L. minor*.

CHAPTER 5: TEMPERATURE EFFECTS ON UPTAKE, PLANT ACTIVITY, AND SORPTION IN *LEMNA MINOR* SYSTEMS

Introduction

Aquatic vegetation is an active component of constructed wetlands. Plants act as filters and sorbents, promote biofilm growth, provide organic carbon to wetland sediments, and remove nutrients like phosphorus and nitrogen. As constructed wetlands are increasingly used to treat municipal wastewaters that contain persistent organic pollutants, the role of plants in actively removing organic pollutants from wastewaters is of increasing importance.

Lemnaceae, a key aquatic plant in many constructed wetlands, actively uptakes persistent organic pollutants from water. Uptake of organic pollutants by Lemnaceae relies on both abiotic and plant-driven processes. Uptake is preceded by sorption and abiotic partitioning of pollutants into plant cells. However, continued uptake depends on plant metabolism of pollutants. For example, *Lemna minor* conjugates 2,4-dichlorophenol with carbohydrate molecules, forming complex products that are sequestered into cell walls and vacuoles (Day and Saunders 2004). Sequestration of pollutants by *L. minor* allows for rapid removal from aqueous phases and introduction of sequestered pollutants into carbon-rich anaerobic sediments at plant death. Studies have indicated that sequestered pollutants are then degraded in anaerobic sediments by microorganisms, indicating that uptake by *L. minor* is an essential component in wetland mineralization of pollutants (Tront et al. 2006). Dependence of uptake on experimental parameters reflects the dual (abiotic and plant-driven) nature of uptake; for example, uptake depends on partitioning properties, such as the fraction of pollutant protonated in aqueous solution, and on metabolic parameters, such as oxygen production rate (Tront and Saunders 2006).

Because temperature can affect both abiotic and plant-driven processes, this study aimed at determining the effects of temperature on uptake of organic pollutants by Lemnaceae. Effects of temperature on sorption and inhibition were also examined to provide insight on the mechanisms behind temperature effects on uptake.

Materials and methods

Lemnaceae was collected from a treatment wetland located near Atlanta, GA, USA that is operated by the Clayton County Water Authority. This wetland is used to polish secondary wastewaters prior to discharge into the raw-water reservoir for the potable-water system of Clayton County, GA, USA. Lemnaceae was visually identified as *Lemna minor*. Prior to experiments, *L. minor* was thoroughly rinsed with distilled water and massed, where mass was determined after removal of excess water through a brief air-drying period and blotting.

L. minor (8 or 11 g/L) was exposed to organic pollutants in triplicate batch reactors containing nutrient medium for 50 h under constant light. Control reactors containing *L. minor* that were inactive due to culture under dark conditions were used to quantify sorption, where sorption included absorption, adsorption, and abiotic partitioning into plant cells. Aqueous concentration of organic pollutant was regularly quantified with HPLC-DAD-MS. After exposure to organic pollutants, the activity of *L. minor* was quantified using an oxygen production rate (OP) assessment developed previously (Reinhold and Saunders 2006a). Inhibition of *L. minor* was determined by normalizing the OP of exposed *L. minor* to control OP of non-exposed *L. minor*.

Experimental pollutants included seven fluorophenols and three trifluoromethylphenols. Initial concentrations ranged from 10 μ M to 500 μ M. The effects of temperature on uptake and sorption were determined for temperatures ranging from 12°C to 35°C (53.6°F to 95°F). This temperature ranged represented summer temperatures typically observed in temperate regions.

Results and Discussion

Uptake of fluorinated organic pollutants

Removal of fluorinated organic pollutants in experimental reactors was rapid; for reactors at 22°C with 10 μ M pollutant, removal levels in 50 h ranged from 21% to 82%. However, removal levels in dark control reactors under similar conditions were substantially less and ranged from -7% to 32%. Headspace oxygen concentrations indicated that after 4 h in darkness, the photosynthetic activity of *L. minor* had ceased and heterotrophic processes were consuming oxygen (Figure 5.1a). Therefore, potential sources for removal of fluorinated pollutants in dark reactors included sorption and microbial degradation.

In dark reactors with *L. minor*, normalized concentration was constant after an initial decrease that was attributed to sorption (Figure 5.2). Similar trends have been

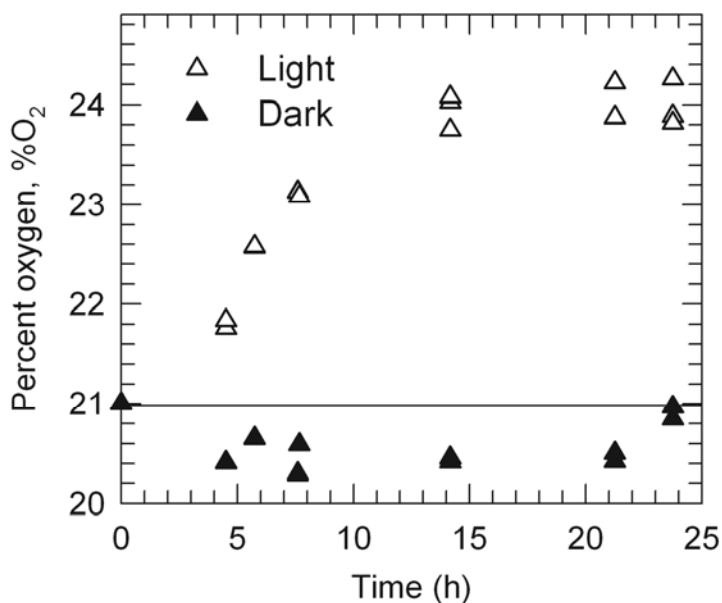


Figure 5.1. Percent oxygen in headspace of sealed reactors containing 0.5 g *L. minor* and 60 mL medium. Solid line represents initial percent oxygen or atmospheric percent oxygen.

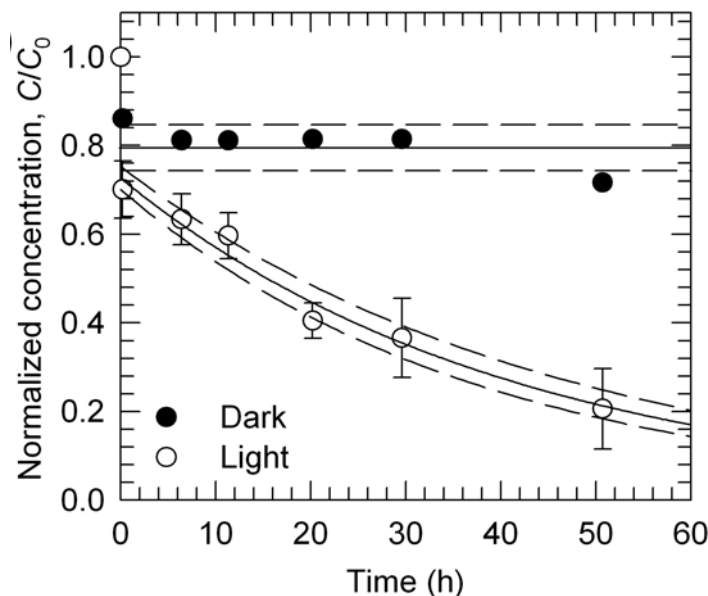


Figure 5.2. Normalized concentration of 2,3-difluorophenol with time in light and dark reactors. Points represent mean and error bars represent standard error of triplicate reactors.

observed for sorption of 2,4,5-trichlorophenol onto chemically-inactivated *L. minor* (Tront and Saunders 2006). In addition to accounting for sorption, dark reactors also indicated an absence of microbial degradation in the reactors systems. The initial decrease observed in the light reactors during the first ten minutes ($C/C_0=0.70\pm0.06$) was statistically similar to average mass of 2,3-difluorophenol sorbed in the dark reactor ($C/C_0=0.81\pm0.05$). However, in contrast to the dark reactors, aqueous phase removal of fluorinated pollutants continued after initial sorption (Figure 5.2). Aqueous removal after the initial sorption phase was attributed to uptake of 2,3-difluorophenol by *L. minor* and was fit with a two-parameter first-order decay relationship that was characterized by a first-order uptake rate coefficient, k ($C/C_0=0.727 \exp (-0.024 t)$). Uptake by *L. minor* was not an isolated phenomenon; previous studies have indicated that 23 of 26 halogenated phenols are rapidly removed from water by *L. minor* (Reinhold and Saunders 2006b; Tront et al. 2007). Uptake rate coefficients ranged from 0.21 d^{-1} to 0.86 d^{-1} for fluorinated pollutants, indicating that removal of fluorinated pollutants by *L. minor* is

likely substantial in many wetlands.

Temperature effects on sorption

The sorption phase of uptake was more closely examined for 3-fluorophenol (3-FP) and 3-trifluoromethylphenol (3-TFMeP) in Figure 5.3. While the mass of 3-FP sorbed was constant over concentrations of 10 μM to 500 μM , the mass of 3-TFMeP sorbed increased linearly with increasing concentration. Increased sorption of high concentrations of 3-TFMeP (as compared to 3-FP) may be related to many physicochemical parameters, including hydrophobicity, solubility, size, and charge rejection. At concentrations less than 100 μM , masses of 3-FP and 3-TFMeP sorbed to 0.5 g *L. minor* in 60 mL medium were similar, despite a substantial difference in hydrophobicity. The log of the octanol-water partitioning coefficients ($\log K_{\text{OW}}$) for 3-

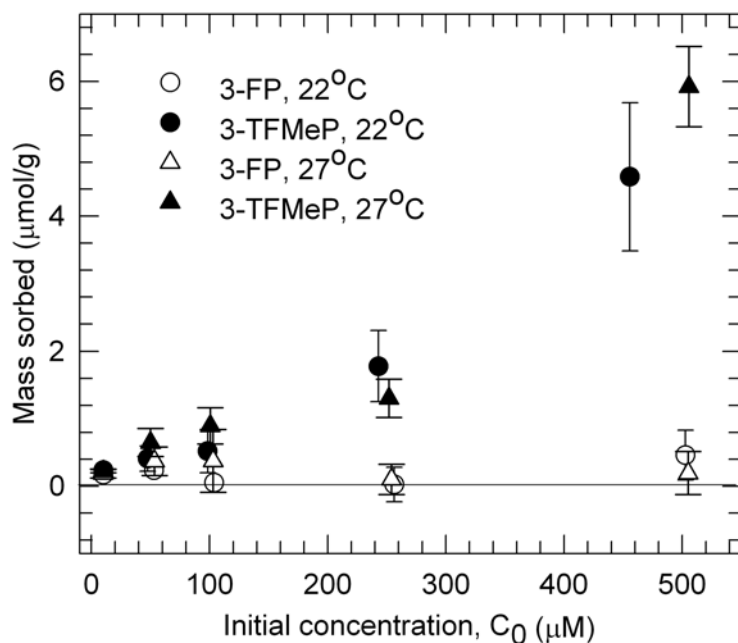


Figure 5.3. Mass of 3-fluorophenol (3-FP) and 3-trifluoromethylphenol (3-TFMeP) sorbed to *L. minor* versus initial concentration at 22°C and 27°C. Points represent mean and error bars represent standard error of triplicate reactors.

FP and 3-TFMeP are 1.93 and 2.95, respectively. A similar trend was also observed for the mass of di- and trifluorophenols sorbed in dark reactors with inactive *L. minor*. For initial concentrations of 10 μM , the mass of fluorinated pollutant sorbed to *L. minor* in dark reactors was independent of $\log K_{\text{OW}}$ for $\log K_{\text{OW}}$ values ranging from 1.7 to 2.5 ($r^2=0.07$; data not shown). Independence of mass sorbed on $\log K_{\text{OW}}$ may indicate that important process that can not easily be differentiated from sorption onto plant tissues. The lack of relationship between mass sorbed at concentrations less than 100 μM and $\log K_{\text{OW}}$ indicates that $\log K_{\text{OW}}$ may not be a good parameter to predict mass sorbed to plant material in wetlands where concentrations are in the nanomolar or parts-per-trillion range. It is important to reiterate that the mass sorbed in this study was the quantification of rapid sorption processes and not the standard sorption experiments that utilize dead plant mass. However, as the mass sorbed in the first ten minutes was similar to the mass sorbed in inactive systems with *L. minor*, these results provide insight into sorptive processes occurring in wetlands.

As relationships between temperature and sorption of organic pollutants depend on both pollutant and sorbent, sorption can increase, decrease, or remain constant with increasing temperature (DiVincenzo and Sparks 2001). The effect of temperature on mass sorbed was examined for temperatures of 12°C to 35°C (Figure 5.4). At an initial concentration of 10 μM , the mass sorbed was constant for the experimental temperatures. However, at concentrations greater than 10 μM 3-TFMeP, the mass sorbed was significantly less at 12°C than at temperatures of 17°C to 35°C. The decrease in mass sorbed in 10 min at 12°C may reflect slower partitioning rates and a requirement for longer exposure times to determine sorption at temperatures less than 17°C. Consequently, this method of determining sorption in active plant systems may be inappropriate for low temperatures. However, at temperatures greater than or equal to 17°C, the 10-min measurement of sorption yielded statistically similar results, indicating that mass sorbed was independent of temperature.

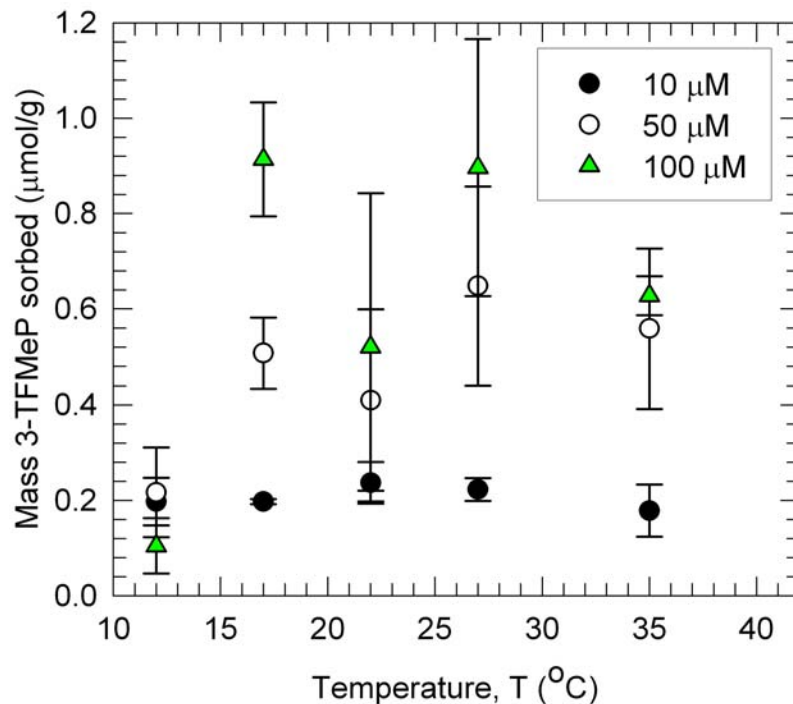


Figure 5.4. Mass of 3-TFMeP sorbed to *L. minor* at temperatures ranging from 12°C to 35°C. Points represent means and error bars represent standard errors of triplicate reactors.

Temperature effects on uptake

In contrast to mass sorbed, uptake rate coefficients increased with increasing temperatures from 12°C to 35°C, as shown in Figure 5.5. The trend between uptake rate coefficients and temperature was represented by a simplified form of the Arrhenius relationship:

$$k = k_{20} \theta^{T-20} \quad \text{Equation 5.1}$$

where: k = uptake rate coefficient at temperature T , d^{-1} ; k_{20} = fitted uptake rate coefficient at 20°C, d^{-1} ; θ = dimensionless temperature coefficient; and T = temperature, °C. Fitted values for k_{20} and θ are provided in Table 5.1. Values were statistically valid with p -values of <0.0001 and r^2 -values of 0.75 or greater. Fitted values for k_{20} and θ were pollutant specific, with greater k_{20} and θ values for trifluoromethylphenols than for

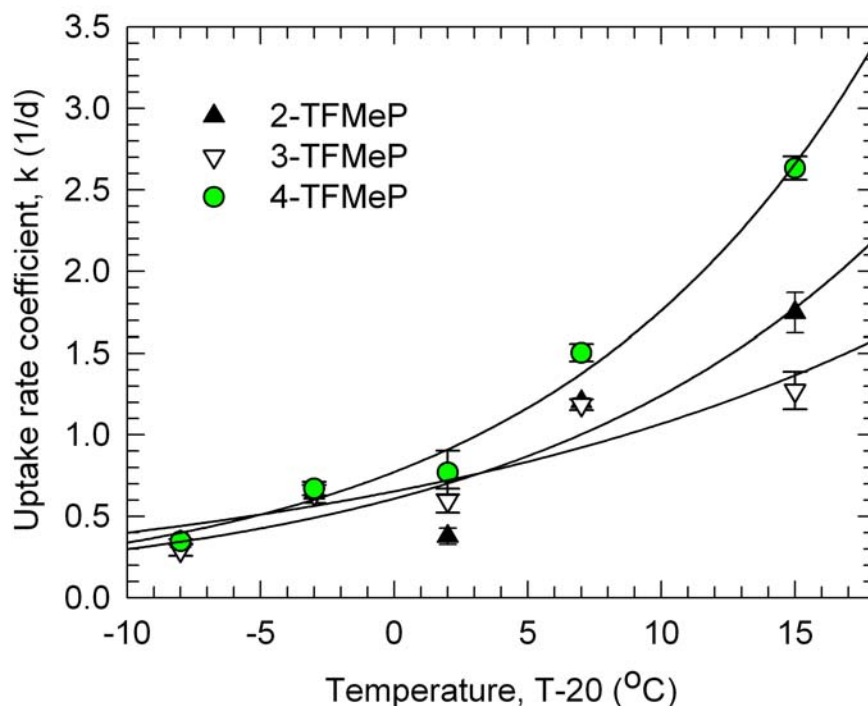


Figure 5.5. Arrhenius relationships for dependence of uptake rate coefficients on temperature for trifluoromethylphenols (TFMeP). Points represent means and error bars represent standard errors of triplicate reactors

Table 5.1. Fitted Arrhenius parameters for uptake of fluorophenols (FP) and trifluoromethylphenols (TFMeP) by *L. minor*. Uptake rate coefficients at 20°C (k_{20}) and temperature coefficients (θ) are listed with standard errors.

	k_{20} (d ⁻¹)	θ
2-FP	0.54 ± 0.02	1.026 ± 0.004
3-FP	0.23 ± 0.01	1.040 ± 0.004
4-FP	0.49 ± 0.05	1.043 ± 0.009
2-TFMeP	0.61 ± 0.07	1.074 ± 0.010
3-TFMeP	0.65 ± 0.06	1.050 ± 0.009
4-TFMeP	0.77 ± 0.04	1.086 ± 0.005

fluorophenols. Temperature coefficients (θ) for fluorophenols ranged from 1.026 ± 0.004 to 1.042 ± 0.009 while temperature coefficients for trifluoromethylphenols ranged from 1.050 ± 0.009 to 1.086 ± 0.005 . The narrow ranges of temperature coefficients for fluorophenols and trifluoromethylphenols may reveal some insight about the enzymatic of processing of fluorinated pollutants by *L. minor*. As the Arrhenius relationship is frequently used to describe temperature dependence of enzymatic reactions, the variation in temperature coefficients reinforces previous studies indicating the strong influence of plant metabolism on the uptake of fluorinated pollutants by *L. minor* (Day and Saunders 2004; Reinhold and Saunders 2006b; Tront and Saunders 2006). Furthermore, independence of mass sorbed on temperature indicates that temperature dependence of uptake was not due to sorption, and, by exclusion, was related to plant-driven metabolism.

Temperature effects on inhibition and plant activity

Temperature was also an important parameter in inhibition of *L. minor* by 500 μM 3-FP or 3-TFMeP. At 22°C, 50% inhibition of OP by *L. minor* occurred at 675 ± 37 μM 3-TFMeP; however, OP was unaffected by concentrations of 3-FP less than 1750 μM (Reinhold and Saunders 2006a). Therefore, 500 μM 3-TFMeP was slightly inhibitory and 500 μM 3-FP was not inhibitory to *L. minor* OP at 22°C. As shown in Figure 5.6, normalized OP indicated that 3-FP did not inhibit *L. minor* at temperatures of 17°C to 27°C. However, *L. minor* was inhibited by 500 μM 3-FP at low (12°C) and high (35°C) temperatures. A similar trend was observed for 3-TFMeP, with the exception that 500 μM 3-TFMeP was also inhibitory at temperatures of 17°C and 22°C. Decreased in normalized OP at 12°C may be related to the substantial decrease in control OP also observed at 12°C, as shown in Figure 5.7. In other words, as plant activity slowed at 12°C, *L. minor* was less able to metabolize and therefore detoxify 3-FP and 3-TFMeP. This explanation is further supported by decreased uptake rates also observed at 12°C, as

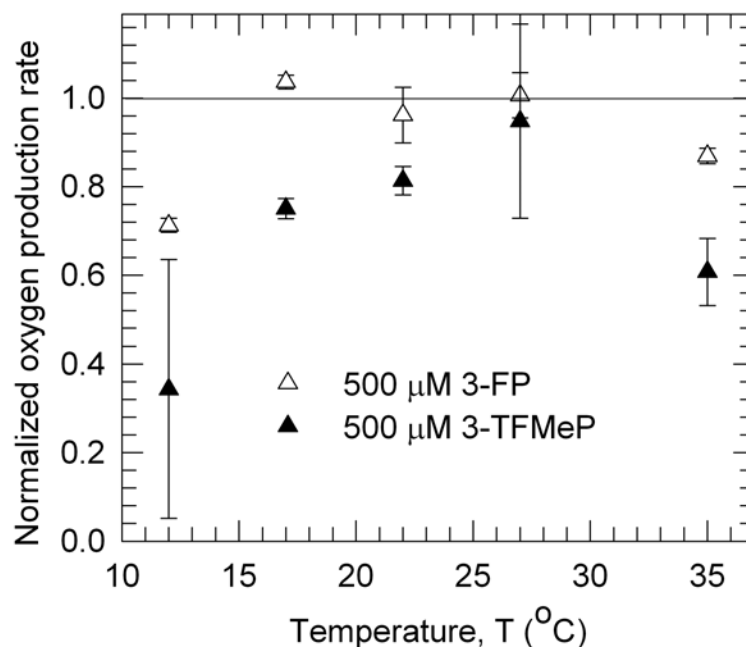


Figure 5.6. Normalized oxygen production rate ($\mu\text{mol/h-g}$) of 0.5 g *L. minor* exposed to 3-fluorophenol (3-FP) and 3-trifluoromethylphenol (3-TFMeP) for 24 h with temperature. Points represent means and error bars represent standard errors of triplicate reactors.

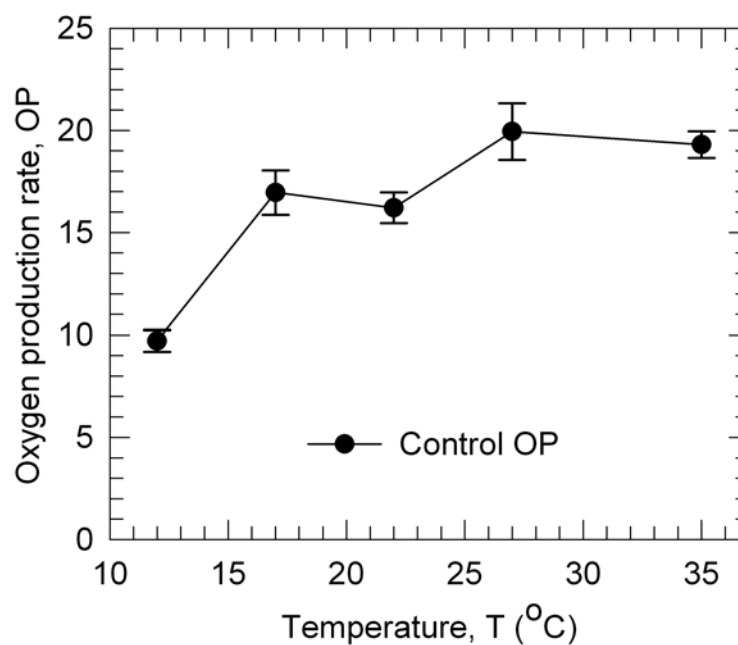


Figure 5.7. Oxygen production rate (OP) in $\mu\text{mol O}_2/\text{h-g}$ *L. minor* of 0.5 g non-exposed *L. minor* with temperature. Points represent means and error bars represent standard deviations of triplicate reactors.

uptake is predominantly driven by plant metabolism. However, this explanation is inappropriate for decreased normalized OP by *L. minor* at 35°C, as control OP was not decreased at 35°C. Additionally, mass sorbed at 35°C was statistically similar for 17°C to 35°C, indicating that increased sorption or partitioning was not responsible for decreased normalized OP at 35°C. Therefore, greater inhibition by 3-FP and 3-TFMeP at 35°C was attributed to an intrinsic susceptibility to toxicity at elevated temperatures. Consequently, multiple mechanisms may account for increased inhibition of *L. minor* at low and high temperatures.

Summary

Aquatic plants are active contributors to treatment of persistent organic pollutants by wetlands. *L. minor* rapidly removed fluorinated organic pollutants, with pseudo-first-order rate constants of 0.21 to 0.86 d⁻¹. Sorption of pollutants onto active plant tissue was independent of log K_{OW} at low concentrations, indicating that partitioning into aqueous phases internal to the plant may be an important component of sorption. Mass of pollutant sorbed to *L. minor* was generally constant with increasing temperature. However, uptake rates increased with increasing temperature. This trend was represented by the Arrhenius relationship and indicated the importance of plant metabolism in uptake (as sorption was constant with temperature). Additionally, the increase in uptake with increasing temperature was not wholly attributable to increased plant activity. While increased inhibition of 3-FP and 3-TFMeP could be attributed to decreased plant activity at 12°C, this explanation was not reasonable for the observed increased inhibition at 35°C. Overall, the temperature effects on mass sorbed and uptake of pollutants by *L. minor* indicates the importance of considering both abiotic and plant-driven processes when designing wetlands to treat persistent organic pollutants. This study demonstrated that active uptake of pollutants by aquatic plants is a relevant and substantial process occurring in treatment wetlands.

CHAPTER 6: UPTAKE OF WASTEWATER-ASSOCIATED ORGANIC POLLUTANTS BY LEMNACEAE

Introduction

Numerous organic chemicals, including pharmaceuticals, personal care products, and pesticides, present growing hazards to natural resources and human and ecosystem health as these organic chemicals are introduced into water systems as organic pollutants. Prevalence of trace concentrations of organic pollutants in U.S. surface waters is wide spread (Kolpin et al. 2002; Boyd et al. 2003; Anderson et al. 2004). Presence of bioactive pharmaceuticals and antimicrobial personal care products, such as ibuprofen, fluoxetine (Prozac®), and triclosan, in surface waters prompts concerns about ecological toxicity, bioaccumulation, and human health hazards, especially when considering life-long exposure concerns on human health. For example, pharmaceutical mixtures that were representative of those found in natural waters inhibited growth of human embryonic liver cells (Pomati et al. 2006).

Effluent from municipal wastewater treatment plants is a major source of organic pollutants in the environment (Kolpin et al. 2002; Ternes et al. 2004). Wetlands may present opportunities for sustainable tertiary treatment and reclamation of wastewaters that contain wastewater-associated organic pollutants. In addition to removing nutrients and polishing residual biodegradable organic matter (e.g., BOD), wetlands have unique capabilities to remove many organic pollutants present in surface waters at trace concentrations. For example, pilot-scale wetlands removed up to 80% of ibuprofen; in contrast, clofibric acid, a lipid-regulating drug, was recalcitrant in pilot-scale wetlands (Matamoros et al. 2005).

Research has indicated that Lemnaceae plants (e.g., *Landoltia punctata* and *Lemna minor*) uptake and metabolize many organic pollutants, including chlorinated

phenols (Day and Saunders 2004; Tront et al. 2007) and fluorinated phenols (Reinhold and Saunders 2006b). However, role of plants in removing many organic pollutants from wastewaters is unknown, and uptake rates, plant metabolite destinations, and ultimate fate of these organic pollutants in wetland systems remain undetermined. Primary aim of research presented herein was to identify plant-associated processes responsible for aqueous depletion of select wastewater-associated organic pollutants to guide future research on fate of organic pollutants in wetland systems. Additionally, research aimed to differentiate between active and passive plant processes that may contribute to fate of organic pollutants and to explore potential relationships between organic pollutant hydrophobicity and aqueous depletion in aquatic plant systems. Objectives were addressed using batch reactors and controls to assess aqueous persistence of six organic pollutants, including pharmaceuticals, pesticides, and personal care products, in aquatic plant systems.

Materials and methods

Collection and maintenance of Lemnaceae

Duckweed, particularly members of the Lemnoideae subfamily of Lemnaceae, was collected from a tertiary wastewater-reclamation wetland located near Atlanta, GA and operated by Clayton County Water Authority (CCWA; Jonesboro, GA). Species were identified using 10 to 40x light and phase contrast microscopy and an internet dichotomous key (Armstrong 2001). Species identification indicated that *Lemna minor* and *Landoltia punctata* were dominant Lemnaceae species in CCWA wetlands; however, *Spirodela polyrhiza* was also occasionally present as a member of Lemnaceae communities dominated by *L. minor* or *L. punctata*. *Wolffia* species, members of the Wolffioideae subfamily of Lemnaceae, were also present as members of Lemnaceae communities and were separated from species of the Lemnoideae subfamily using a No.

10 U.S.A. Standard testing sieve with 2.0 mm openings. Prior to disinfection, Lemnaceae communities were maintained in Standard Methods medium (APHA et al. 1998b) under a combination of fluorescent and plant growth lights. Experiments utilized Lemnaceae that had acclimated to laboratory conditions for a minimum of three days.

Chemicals and stock preparations

Experimental pollutants are listed in Table 6.1, with physicochemical properties and solvents used for stock preparations. Chemical structures of experimental pollutants are provided in Figure 6.1. Experimental organic pollutants were purchased through VWR (West Chester, PA). E-pure water was generally used for stock preparation; however, methanol was used for preparation of triclosan stocks because of low aqueous solubility.

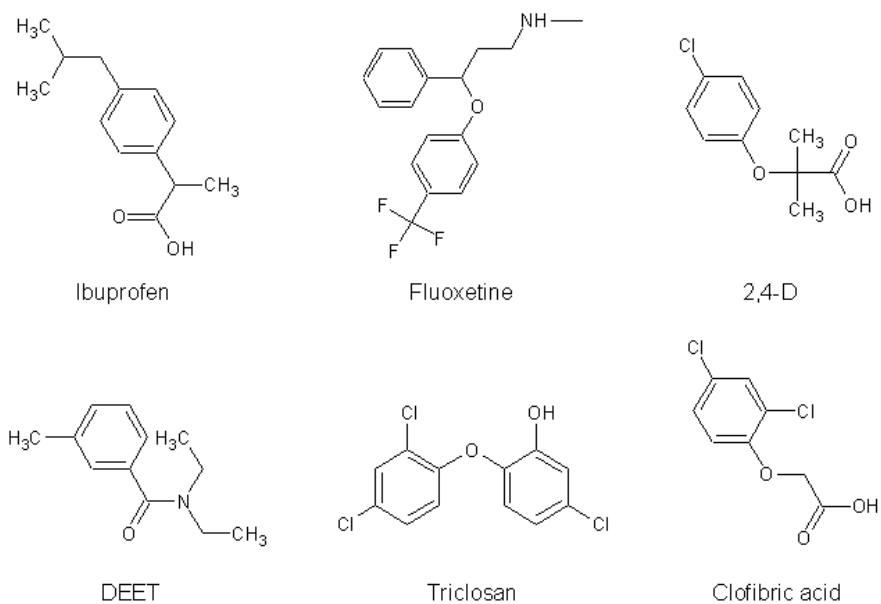


Figure 6.1. Chemical structures of experimental organic pollutants.

Table 6.1. Physicochemical properties of experimental organic pollutants and solvents used for stock preparations. Aqueous solubility and pK_a values are from SciFinder Scholar: ACD/Labs Software V8.14 for Solaris. Octanol-water partitioning coefficients (log K_{OW}) values are experimental values provided by EPI Suite v. 3.12.

Pollutants	Use	Chemical name	Mol wt.	Aq. Sol. (g/L)	Log K_{OW}	pK_a	Stock solvent
Ibuprofen	Pharmaceutical	2-[4-(2-methylpropyl)phenyl] propanoic acid	206.3	19.0	3.97	4.41	H ₂ O
Fluoxetine	Pharmaceutical	N-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy]propan-1-amine	309.3	38.4	4.05	10.05	H ₂ O
Clofibric acid	Pharmaceutical	2-(4-Chlorophenoxy)-2-methylpropanoic acid	214.6	1000	2.57	3.18	H ₂ O
DEET	Personal care product	Meta-N,N-diethyl toluamide	191.3	0.67	2.18	NA	H ₂ O
Triclosan	Personal care product	5-chloro-2-(2,4-dichlorophenoxy)-phenol	289.5	0.004	4.76	8.1	MeOH
2,4-D	Pesticide	2-(2,4-dichlorophenoxy) acetic acid	221.0	999	2.81	2.98	0.1 N NaOH

Experimental reactors and controls

Aqueous depletion of experimental organic pollutants was determined using single-solute batch reactors and controls. Reactors and controls consisted of 250-mL Erlenmeyer flasks with 100 mL of Standard Methods (SM) medium (APHA et al. 1998b). An additional 10 mM of phosphate buffer was added to SM medium to minimize pH change (Reinhold and Saunders 2006a). Initial concentrations of experimental organic pollutants were 10 μ M; additionally, initial concentrations of 5 μ M fluoxetine were used due to inhibition of Lemnaceae oxygen production rates at 10 μ M fluoxetine. Experimental reactors and controls were maintained under constant light at $25.0 \pm 0.2^\circ\text{C}$. Tested in triplicate, experimental reactors and controls were consisted of:

Active Lemnaceae reactors. Active Lemnaceae reactors contained 2.0 g of either *L. punctata* or *L. minor*. Active Lemnaceae reactors utilized full surface coverage of Lemnaceae and aluminum-foil sidewalls to minimize light penetration into aqueous medium and volatilization from water-air interface.

Sorption controls. Sorption controls contained 2.0 g of Lemnaceae that had been inactivated by exposure to 1 g/L sodium azide for 3 – 7 d. Similar Lemnaceae communities (i.e., same species and collection source and date) were used in sorption controls and active Lemnaceae reactors to facilitate quantitative comparisons between sorption controls and active Lemnaceae reactors. Sorption controls also had aluminum-foil sidewalls and full surface coverage of Lemnaceae.

Microbial degradation controls. Lemnaceae (2.0 g per each reactor) was macerated in a laboratory-grade blender for 120 seconds to eliminate plant activity while presumably maintaining active plant-associated microbial communities. Similar Lemnaceae communities (i.e., same species, collection source, and collection date), and thus similar microbial communities, were used in microbial degradation controls and active Lemnaceae reactors. Microbial degradation controls were qualitatively compared

with active Lemnaceae reactors, as maceration increased surface area of Lemnaceae available for sorption. Additionally, light penetration into microbial degradation controls, even with aluminum-foil sidewalls, was substantially greater than in active Lemnaceae reactors. Consequently, microbial degradation controls were quantitatively compared to active Lemnaceae reactors only when sorption and physicochemical loss processes did not affect organic pollutant concentration.

Physicochemical loss controls. Physicochemical loss controls consisted of modified SM medium, experimental organic pollutant, no Lemnaceae, and aluminum-foil sidewalls. As surface coverage of Lemnaceae in active Lemnaceae reactors and sorption in controls minimized light penetration and medium surface area available for volatilization, physicochemical loss controls were used for qualitative, not quantitative, identification of physicochemical processes that contributed to loss in active Lemnaceae reactors, sorption controls, and physicochemical loss controls.

Assessments of aqueous depletion processes for organic pollutants were conducted with both *L. minor* and *L. punctata* populations, with exceptions of DEET (*L. minor* only) and clofibric acid (*L. punctata* only). Selection of Lemnaceae species for given assessment was based on presence of species at CCWA wetlands during collection immediately prior to assessment and robust growth under laboratory conditions after collection.

Assessments utilizing *L. minor* were conducted during spring and early summer (2006 and 2007), while assessments utilizing *L. punctata* were conducted year round (June 2006 to July 2007).

Based on results from initial assessments of aqueous depletion processes, subsequent studies were completed with ibuprofen and fluoxetine. Role of plant activity in aqueous depletion of ibuprofen was explored by assessing aqueous depletion in active duckweed reactors containing range of nutrient concentrations (i.e., 0, 0.5, 1.0, 1.5, and 2.0 times concentrations in SM medium). Supplemental experiments with fluoxetine included alternative microbial degradation controls. Lemnaceae, 1.5 g per control, was

added to 60-mL of modified SM medium in 160-mL serum bottles. Triplicate controls were sealed and maintained in darkness, through full coverage of bottles with black fabric and storage in dark boxes, for 8 h. Care was taken to maintain dark conditions upon addition of stock fluoxetine and during sampling events. For comparison purposes, alternative active Lemnaceae reactors and sorption controls, using 1.5 g of Lemnaceae and 60-mL modified medium in 160-mL serum bottles, were prepared and sampled in parallel with alternative microbial degradation controls.

Experimental reactors and controls were sampled prior to, and immediately after, addition of Lemnaceae to reactors and controls. Aqueous samples were taken four to six times in the first 48-h, daily from day 2 to 7, and every other day from day 7 for the duration of each experiment. Experiments ranged from 5-d to 26-d exposures. Experimental organic pollutants were screened for sorption to sample preparation filters prior to experiments. Fluoxetine substantially sorbed to all filters, including PTFE, cellulose acetate, and GHP filters, except for aluminum oxide filters. Sorption of remaining experimental organic pollutants to PTFE filters was minimal and constant filtrate concentrations were observed for 1 mL to 5 mL. Consequently, aqueous samples were filtered through 0.2 μ M Whatman PTFE filters, with the exception of fluoxetine samples, which were filtered through 0.2 μ M Whatman aluminum oxide Anodisk filters.

Samples were analyzed on an Agilent (Palo Alto, CA, USA) Model 1100 liquid chromatography (LC) system equipped with a UV/Vis diode array detector (DAD) and mass spectrometer (MS). Experimental organic pollutants were separated from medium components using isocratic separation with acetonitrile and 0.1% acetic acid in water on a reverse phase STABLE BOND-C18 analytical column (2.1x150 mm, 5 μ m, Agilent). Presence and/or absence of aqueous metabolites, microbial degradates, or physicochemical products were monitored using DAD at 210 nm and electrospray ionization (ESI) in negative mode on MS.

With a few exceptions, oxygen production rate of Lemnaceae in active

Lemnaceae reactors was measured at conclusion of experiments (Reinhold and Saunders 2006a); details of oxygen production rate assessment and inhibition comparisons have been previously described (Chapter 4) and published (Reinhold and Saunders 2006a) in detail. Oxygen production rates were not measured in experiments where substantial senescence of Lemnaceae plants occurred during >2-wk exposure to organic pollutant, as sufficient plant mass (i.e., 1.0 g per reactor) was not available. Oxygen production rates of Lemnaceae exposed and not exposed to experimental organic pollutants were compared to assess inhibition of plant activity due to organic pollutant exposure. If inhibition of Lemnaceae oxygen production rate was observed at initial concentration of experimental organic pollutant, experiments were repeated with lower initial concentrations of experimental organic pollutant (e.g., 5 μ M fluoxetine). Additionally, oxygen production rate assessment was used to quantify plant activity at conclusion of ibuprofen experiments with varied media concentrations.

Results and discussion

Assessments of plant-associated aqueous depletion processes in active Lemnaceae reactors and controls yielded valuable information as to identifying important plant-associated processes responsible for aqueous depletion of organic pollutants. Results are discussed in detail below for each organic pollutant; however, a brief overview of the ability of experimental methodology to distinguish aqueous depletion processes is provided here.

In physicochemical loss controls, photodegradation and hydrolysis were distinguished from volatilization based on detection of photodegradation products in aqueous medium. It is important to note that physicochemical losses also occurred in active Lemnaceae reactors, sorption controls, and microbial degradation controls; however, physicochemical losses in active Lemnaceae reactors and sorption controls were limited by full surface coverage of Lemnaceae and coverage of reactor and control

sidewalls with foil (e.g., minimizing available air-water interface for volatilization and minimizing light penetration into water column). Photodegradation, distinguished by identification of aqueous photodegradation products, was observed for one experimental organic pollutant (e.g., triclosan).

Aqueous depletion was observed in microbial degradation controls, but not in physicochemical loss controls, for two experimental organic pollutants, indicating that microbial degradation of organic pollutants was observable in microbial degradation controls. However, microbial degradation products were only identified for one of two organic pollutants, suggesting that inability to detect microbial degradation products and elucidate degradation pathways for some organic pollutants may limit utilization of microbial degradation controls. Inability to detect microbial degradation products likely resulted from sorption of products to macerated plant tissues; additionally, microbial degradation controls were unsuccessful for 2 of 6 organic pollutants because of immediate and complete sorption of organic pollutants to macerated tissues. Sorption of organic pollutants to macerated plant tissues necessitated use of alternative microbial degradation controls, which consisted of closed serum bottles with Lemnaceae that were maintained in complete darkness.

Sorption controls were essential in identifying presence of plant uptake of organic pollutants, as light conditions in sorption controls were similar to those of active Lemnaceae reactors. However, identification of plant uptake as important aqueous depletion process for organic pollutants in wetlands was limited to pollutants that were not removed in microbial degradation controls. Subsequent studies examining relationship between organic pollutant aqueous depletion and plant activity were useful in determining whether active plant processes attributed to aqueous depletion when microbial degradation was observed.

Results were reproducible; while rates of aqueous depletion were not quantified, results from multiple trials for each organic pollutant yielded similar conclusions as to

identification of plant-associated processes that contributed to aqueous depletion. Additionally, assessment results were not affected by Lemnaceae species or collection date, and similar results were observed for both *L. minor* and *L. punctata* populations.

Ibuprofen

Concentration of ibuprofen decreased in active Lemnaceae reactors and microbial degradation controls, but was constant in sorption and physicochemical loss controls, as shown in Figure 6.2. In active Lemnaceae reactors, concentration of ibuprofen was constant for a lag period of typically 20 to 24 hr and then decreased linearly, resulting in $47.5 \pm 3.9\%$ aqueous depletion in 9 d. Aqueous depletion in microbial degradation controls was slower than aqueous depletion in active Lemnaceae reactors, with $24.8 \pm 2.0\%$ depletion in 9 d. Aqueous depletion of ibuprofen in microbial degradation controls suggested that microbial degradation attributed to aqueous depletion of ibuprofen in active Lemnaceae reactors; however, greater aqueous depletion in active Lemnaceae reactors than in microbial degradation controls warranted further examination of potential plant-associated processes that could contribute to aqueous depletion of ibuprofen.

An unknown peak was consistently observed in samples from active Lemnaceae reactors and microbial degradation controls. This peak was further investigated using ESI in the negative mode. The negative mass spectra of this peak consisted of a single m/z ratio of 221.1, indicating that the unknown peak was likely hydroxy-ibuprofen, a known microbial degradation product of ibuprofen with a m/z ratio of 221 under negative ESI (Quintana et al. 2005). Structures and molecular weights of ibuprofen and two isomers of hydroxy-ibuprofen are provided in Figure 6.3. Two isomers of hydroxy-

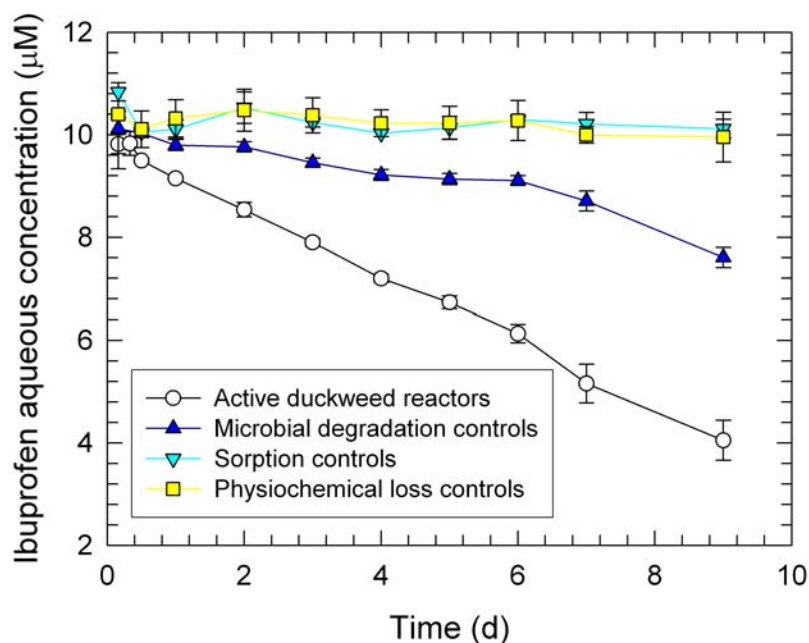


Figure 6.2. Aqueous concentration of ibuprofen with time in experimental reactors and controls. Error bars represent standard deviation of triplicate reactors. Active Lemnaceae reactors, microbial degradation controls, and sorption controls utilized *L. punctata* populations. Points represent means and error bars represent standard errors of triplicate reactors.

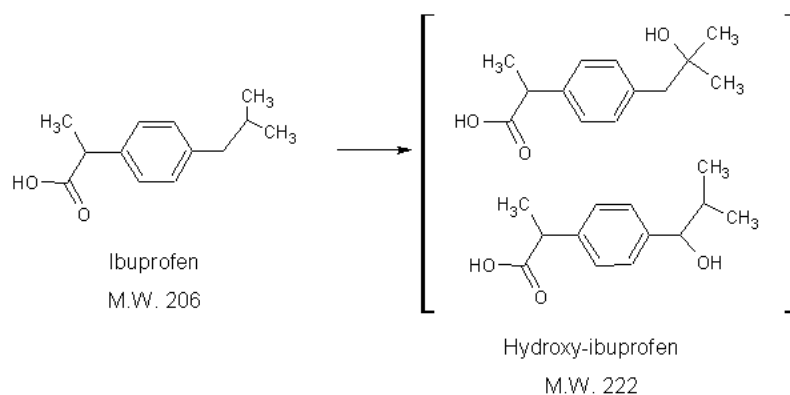


Figure 6.3. Chemical structures and molecular weights (M.W.) of ibuprofen and two isomers of hydroxy-ibuprofen.

ibuprofen, 1-hydroxy-ibuprofen and 2-hydroxy-ibuprofen, and carboxy-ibuprofen have been identified as initial microbial degradation products of ibuprofen by Zwiener et al. (2002) and Quintana et al. (2005). One isomer of hydroxy-ibuprofen was the only product consistently observed in active Lemnaceae reactors and microbial degradation controls. Absence of other microbial degradation products may have resulted from decreased microbial density and/or diversity associated with active Lemnaceae reactors, as previous studies examined degradation by microbial communities from activated sludge. However, results indicated that microbial communities associated with Lemnaceae are able to degrade ibuprofen.

Microbial transformation of ibuprofen to hydroxyl-ibuprofen was monitored in active Lemnaceae reactors and microbial degradation controls for 26 d (Figure 6.4). Ibuprofen was completely removed by 15 d in active Lemnaceae reactors and by 26 d in microbial degradation controls. Concentration of hydroxy-ibuprofen, as indicated by DAD response at 210 nM, increased proportionally with decrease in ibuprofen concentration in both active Lemnaceae reactors and microbial degradation controls. For example, when ibuprofen concentration was $2.4 \pm 0.3 \mu\text{M}$ in active Lemnaceae reactors at 9 d, relative concentration of hydroxy-ibuprofen was 43.4 ± 3.3 area in milliamp units (AMAU); likewise, when ibuprofen concentration was $2.5 \pm 0.2 \mu\text{M}$ in microbial degradation controls, relative concentration of hydroxy-ibuprofen was 39.9 ± 3.0 AMAU. Proportional increase of hydroxy-ibuprofen with decrease in ibuprofen further supported identification of this product as a metabolite of ibuprofen active Lemnaceae reactors and microbial degradation controls. Concentration of hydroxy-ibuprofen decreased in active Lemnaceae reactors after ibuprofen concentrations were depleted at 15 d, indicating further degradation of hydroxy-ibuprofen. No additional peaks were observed in aqueous medium of active Lemnaceae reactors after 15 d. Previous studies have indicated that hydroxy-ibuprofen was mineralized by cometabolic microbial degradation (Buser et al. 1999; Quintana and Reemtsma 2004; Quintana et al. 2005). It is important to note that

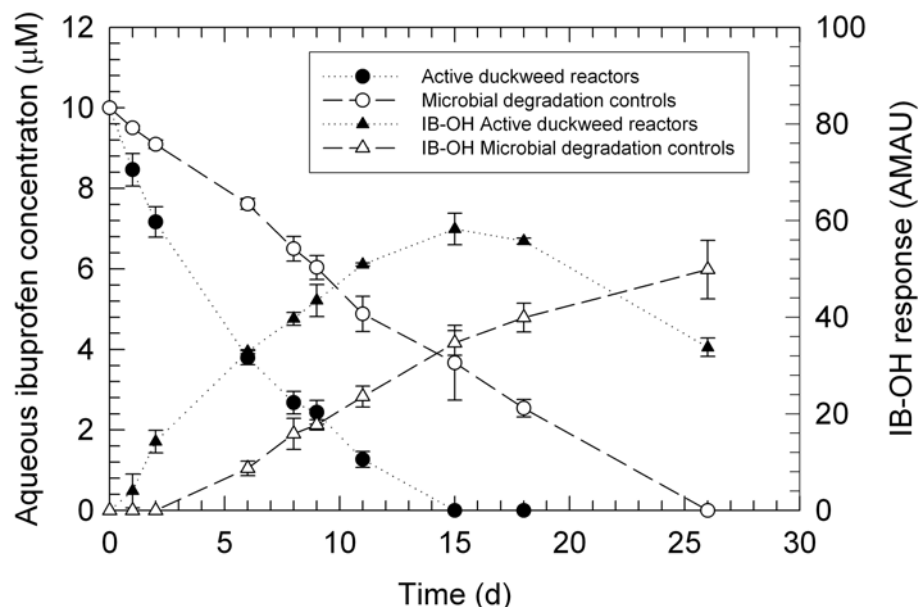


Figure 6.4. Concentration of ibuprofen (left axis) and relative concentration of hydroxy-ibuprofen (right axis) with time in active Lemnaceae reactors and microbial degradation controls. Reactors and controls utilized *L. punctata* populations. Points represent means and error bars represent standard errors of triplicate reactors.

substantial plant death (~50% decrease in Lemnaceae surface area) was observed during the 26 d experiment, reemphazing importance of microbial processes in the aqueous depletion of ibuprofen in both active Lemnaceae reactors and microbial degradation controls.

Relative decrease in ibuprofen concentration, and related increase in hydroxy-ibuprofen concentration, was greater in active Lemnaceae reactors than microbial degradation controls. Similar relative proportions of ibuprofen to hydroxy-ibuprofen in active Lemnaceae reactors and microbial degradation controls indicated that plant uptake of ibuprofen or hydroxy-ibuprofen did not account for increased aqueous depletion of ibuprofen in active Lemnaceae reactors. Instead, results suggested that Lemnaceae plants are supporting increased microbial degradation of ibuprofen, most likely through provision of aqueous organic carbon resulting in enhancement of microbial growth and/or cometabolic degradation of ibuprofen. This hypothesis was tested by further examination

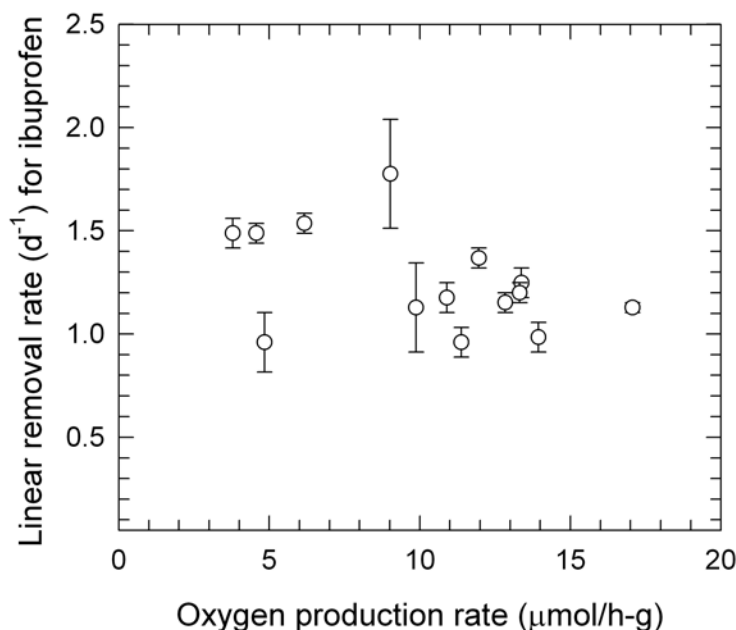


Figure 6.5. Linear aqueous depletion rates for ibuprofen in active reactors for 24 to 120 h versus Lemnaceae oxygen production rate at conclusion of exposure. Error bars represent standard error of fit for first order exponential decay relationships between aqueous concentration (μM) and time (h). Points represent means and error bars represent standard errors of triplicate reactors.

of role of plant activity in aqueous depletion of ibuprofen in active Lemnaceae reactors exhibiting a range of oxygen production rates (e.g., 3.8 – 17.1 $\mu\text{M O}_2/\text{h-g}$ Lemnaceae). As shown in Figure 6.5, there was no relationship between oxygen production rates and aqueous depletion, as represented by linear aqueous depletion rates derived for decreases in concentration of ibuprofen from 24 to 120 h. Previous studies have indicated a positive correlation between oxygen production rate and uptake of 2,4,5-trichlorophenol by Lemnaceae (Tront and Saunders 2006); the absence of a similar correlation for ibuprofen suggested that active plant processes, particularly uptake, did not contribute to aqueous depletion of ibuprofen in active Lemnaceae reactors. Therefore, results are consistent with the hypothesis that Lemnaceae is enhancing microbial degradation of ibuprofen.

Fluoxetine

Concentrations of fluoxetine decreased in active Lemnaceae reactors and in microbial degradation and sorption controls, as shown in Figure 6.6. Inhibition of Lemnaceae oxygen production was observed at both 5 μ M and 10 μ M fluoxetine (i.e., $73.4 \pm 15.0\%$ and $80.2 \pm 7.0\%$ inhibition, respectively). No loss was observed in physicochemical loss controls, indicating limited volatilization and photodegradation of fluoxetine under experimental conditions. Sorption to Lemnaceae accounted for $55.6 \pm 3.9\%$ of aqueous depletion of 10 μ M fluoxetine and $77.7 \pm 0.4\%$ of aqueous depletion of 5 μ M fluoxetine in Lemnaceae reactors, as indicated by the sorption controls. As compared to previous studies with chlorinated phenols (Tront and Saunders 2006), sorption kinetics were relatively slow and sorption equilibrium was observed 12 to 24 h after exposure. Aqueous depletion in active Lemnaceae reactors was greater than aqueous depletion in sorption controls (e.g., $18.1 \pm 4.2\%$ and $44.4 \pm 3.9\%$ greater depletion for 5 μ M and 10 μ M fluoxetine, respectively). While additional aqueous depletion of fluoxetine was likely due to plant uptake, further studies were completed to confirm that microbial degradation was not contributing to aqueous depletion of fluoxetine in active Lemnaceae reactors.

Low recovery of fluoxetine from macerated control necessitated alternative methods to explore the role of microbial degradation in fate of fluoxetine in Lemnaceae systems. As the prolonged exposure of Lemnaceae and associated microorganisms to 1 g/L sodium azide used to create sorption controls would likely disrupt microbial communities, standard sorption controls were not considered good substitutes for microbial degradation controls. Therefore, Lemnaceae plants were inactivated by exposure to prolonged darkness in sealed reactors that were subsequently used to evaluate the role of non-photosynthetic microorganisms in fate of fluoxetine in Lemnaceae systems. Previous studies have indicated that oxygen production by Lemnaceae ceases after as few as 2 h of darkness in sealed reactors

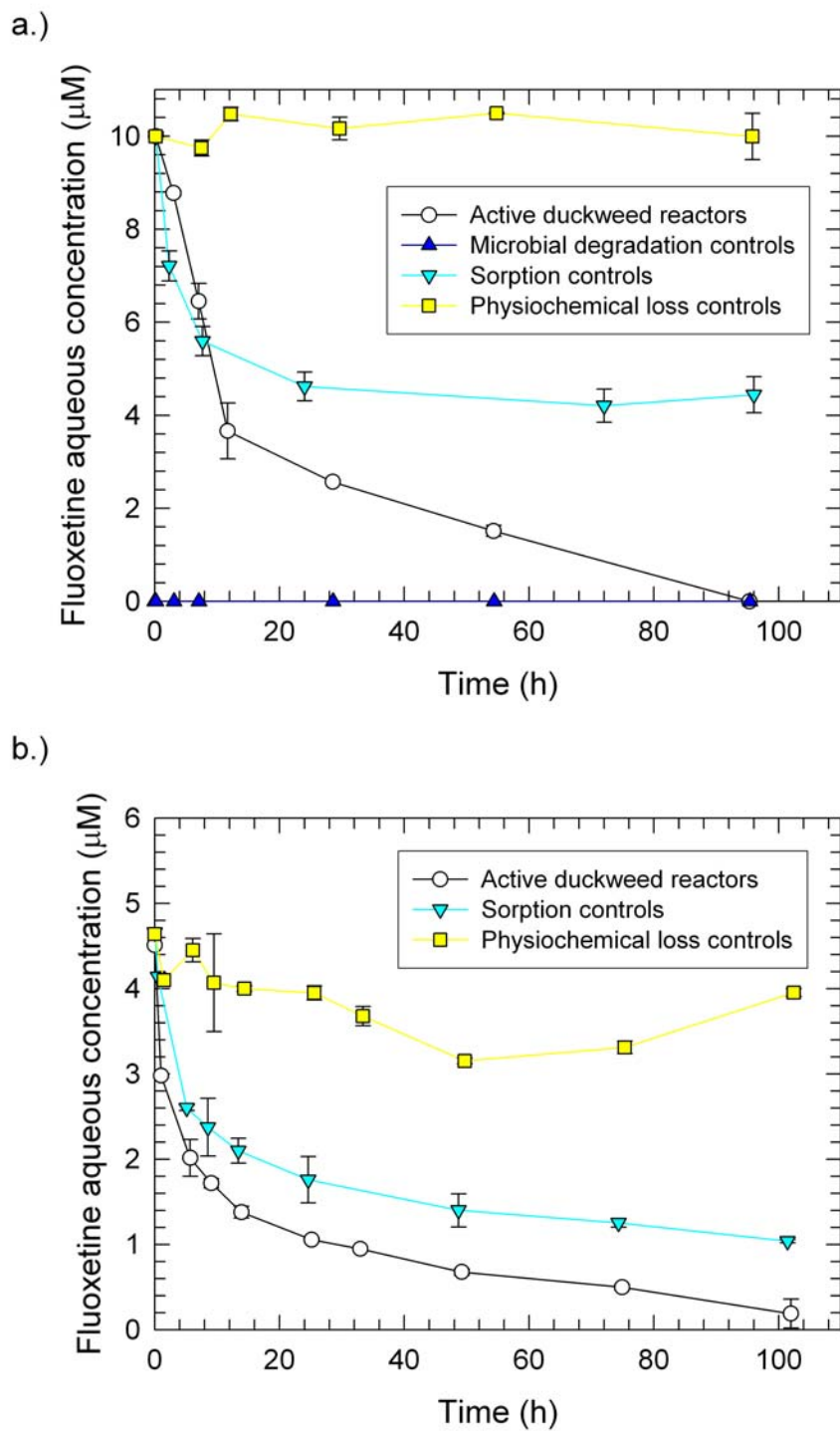


Figure 6.6. Aqueous concentration of fluoxetine with time in experimental reactors with initial concentrations of (a.) 10 μM (*L. punctata*) and (b.) 5 μM (*L. minor*). Error bars represent standard deviations for triplicate reactors.

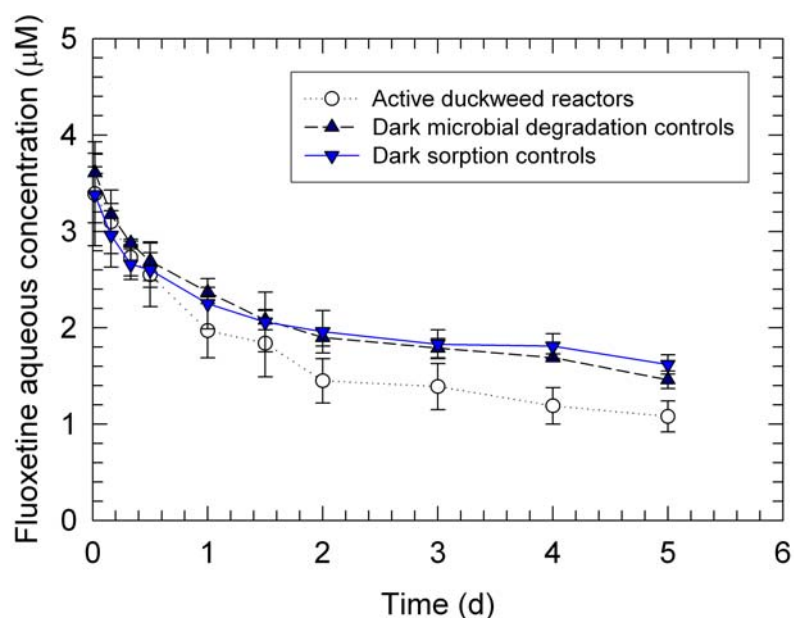


Figure 6.7. Concentration of fluoxetine (μM) with time (d) for active Lemnaceae reactors and control reactors with Lemnaceae inactivated by exposure to darkness (active dark control) or sodium azide (sorption dark control). Error bars represent standard deviations for triplicate reactors.

(Reinhold and Saunders 2006a). As shown in Figure 6.7, aqueous depletion in inactivated Lemnaceae control reactors was independent of method of inactivation, indicating that non-photosynthetic microorganisms do not significantly contribute to aqueous depletion in fluoxetine in Lemnaceae systems. Additionally, slightly more aqueous depletion was observed in active Lemnaceae reactor than in dark Lemnaceae or dark sorption controls, indicating that Lemnaceae actively uptakes fluoxetine.

Sorption to plant tissues was major aqueous depletion process for fluoxetine in active Lemnaceae reactors. Relative contribution of sorption to overall aqueous depletion increased with decreasing concentration (i.e., $55.6 \pm 3.9\%$ of depletion of $10 \mu\text{M}$ fluoxetine vs. $77.7 \pm 0.4\%$ of depletion of $5 \mu\text{M}$ fluoxetine); consequently, relative contribution of plant uptake to aqueous depletion of aqueous fluoxetine in wastewaters with less than nM concentrations of fluoxetine is likely not distinguishable from sorption.

However, it is important to note that active plant uptake occurs in parallel with sorption and, furthermore, that active plant uptake is not an equilibrium process, as is sorption. Sorption of fluoxetine to plant tissues, while contributing to aqueous depletion, would not contribute to mass removal from wetland systems. However, active plant uptake of fluoxetine indicates that plants can metabolize fluoxetine. Plant metabolism of organic pollutants, via transformation, conjugation, and/or sequestration, could contribute to mass removal of organic pollutants from wetland systems. Additionally, active plant uptake has important implications for fate, as uptake can adversely and beneficially affect fate of organic pollutants in wetland systems. Potential negative implications include consumption of sequestered fluoxetine by herbivores, while potential beneficial implications include degradation of fluoxetine by P450 enzymes in plants or transformation and conjugation of fluoxetine to form metabolites with decreased bioactivity and toxicity.

Clofibric acid

Clofibric acid concentrations were generally constant in active Lemnaceae reactors and controls, as shown in Figure 6.8. Initial 5-d experiments indicated slight loss of clofibric in active Lemnaceae reactors and microbial degradation controls (e.g., $3.2 \pm 0.1\%$ and $11.1 \pm 0.2\%$, respectively). However, 16-d exposure of active and macerated Lemnaceae to clofibric acid indicated that loss was negligible in active Lemnaceae reactors and microbial controls.

Meta-N,N-diethyl toluamide (DEET)

DEET concentrations were constant in active Lemnaceae reactors and physicochemical loss controls for 4 d, as shown in Figure 6.9. Aqueous depletion of $13.2 \pm 4.1\%$ of DEET was observed in microbial degradation controls during the first three hours; however, subsequent measurements indicated that concentration of DEET in

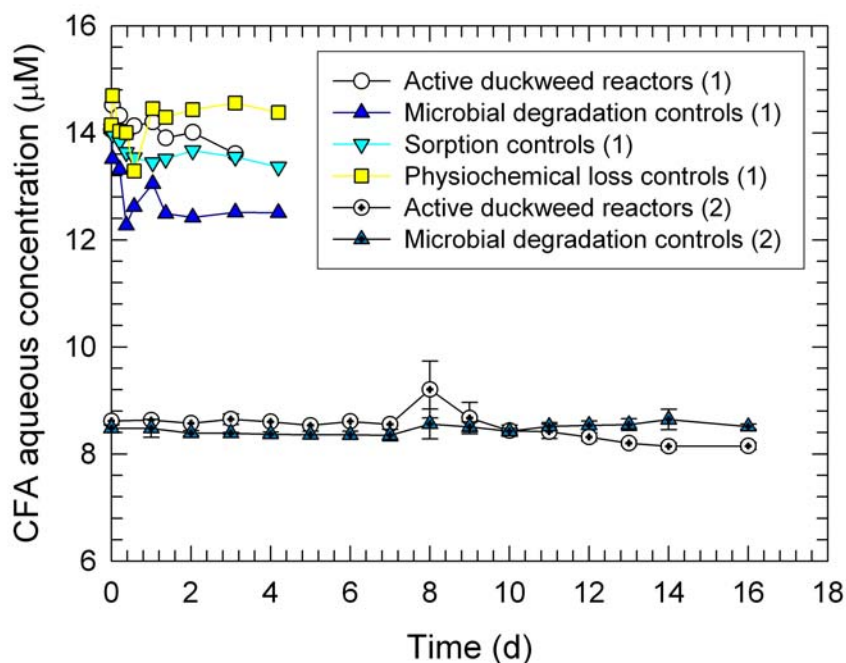


Figure 6.8. Aqueous concentration of clofibric acid (CFA) in reactors over 5 d (Trial 1: *L. minor*) and 16 d (Trial 2: *L. punctata*). Points represent means and error bars, not always visible, represent standard error of triplicate reactors.

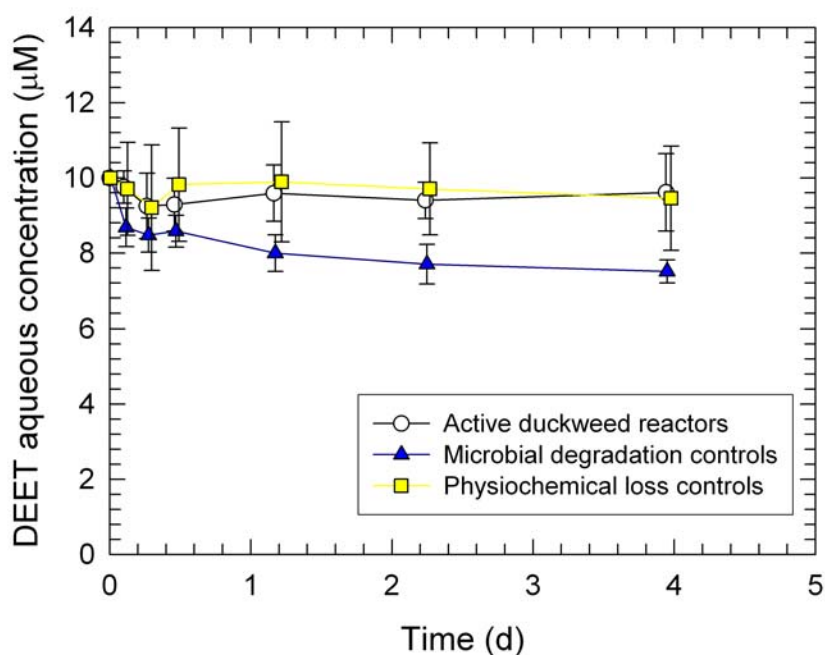


Figure 6.9. Concentration of DEET (μM) with time (d) in active Lemnaceae reactors and microbial degradation and physicochemical loss controls. Error bars represent standard deviations of triplicate reactors.

microbial degradation controls was constant from 3 h to 4 d. Initial aqueous depletion of DEET in microbial degradation controls was attributed to sorption to macerated Lemnaceae tissues. Additionally, microbial degradation of DEET was considered negligible in active Lemnaceae reactors and microbial degradation controls, as concentration of DEET did not decrease over 3 h to 4 d. Consequently, results indicate that DEET was persistent in Lemnaceae systems.

Triclosan

Triclosan concentrations decreased in active Lemnaceae reactors and controls, as shown in Figure 6.10. Immediate aqueous depletion in microbial degradation reactors was attributed to sorption to macerated Lemnaceae. Aqueous depletion in active Lemnaceae reactors and sorption controls were statistically similar on days 0 to 3. After the third day, aqueous depletion in active Lemnaceae reactors was slightly greater than in sorption

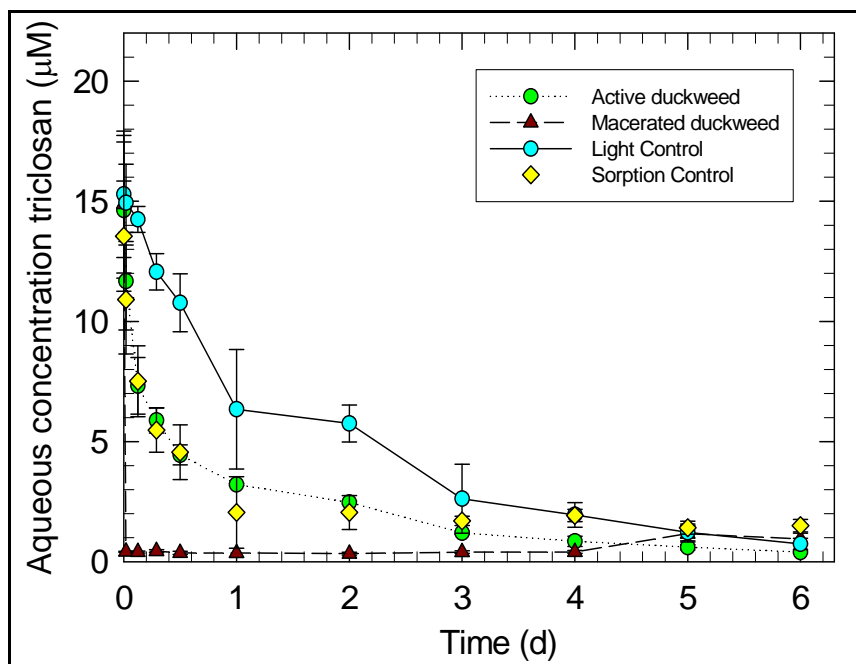


Figure 6.10. Aqueous concentration of triclosan with time in experimental reactors with initial concentration of 15 μM . Points represent means and error bars represent standard errors for triplicate reactors.

controls. Decrease in aqueous concentration of triclosan was also observed in physicochemical loss controls, indicating that photodegradation or volatilization may be an important aqueous depletion process for triclosan in Lemnaceae systems.

Based on previous studies indicating photodegradation of triclosan to chlorinated phenols (Latch et al. 2005), samples from triclosan reactors were monitored for presence of 2,4-dichlorophenol (2,4-DCP), 2-chlorophenol, and 4-chlorophenol. 2,4-DCP was observed in sorption controls and microbial degradation controls, but was not detected in active Lemnaceae controls. Monochlorophenols were not detected in active Lemnaceae reactors or controls. As shown in Figure 6.11, concentration of 2,4-DCP in triclosan sorption controls increased from non-detectable concentrations to $0.52 \pm 0.23 \mu\text{M}$ during the first day of triclosan exposure. Concentrations of 2,4-DCP in triclosan sorption controls subsequently increased from day 1 to day 6, indicating slight accumulation of 2,4-DCP in triclosan sorption controls. Increase in 2,4-DCP was significant from day 2

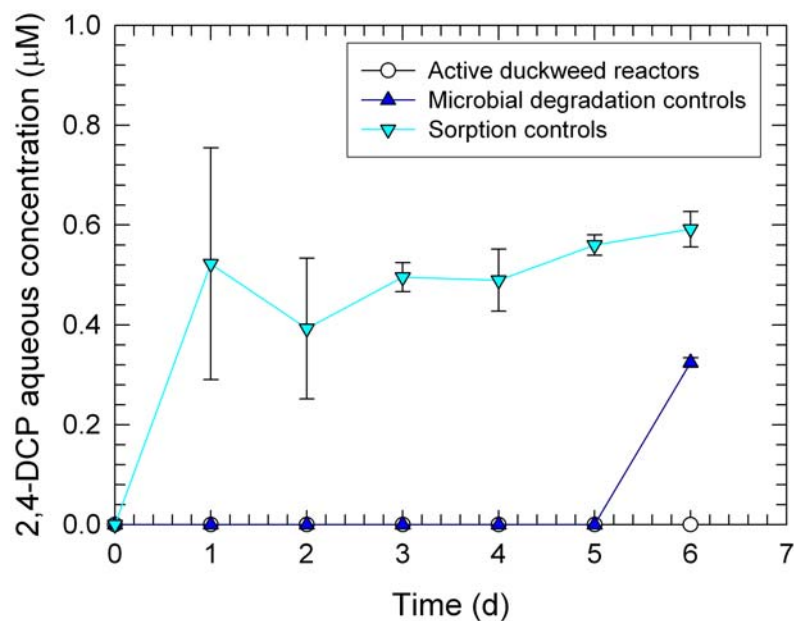


Figure 6.11. Concentration of 2,4-DCP in active Lemnaceae reactors and controls exposed to triclosan. Points represent means and error bars represent standard errors of triplicate reactors.

to day 6 ($0.39 \pm 0.14 \mu\text{M}$ vs. $0.59 \pm 0.04 \mu\text{M}$ 2,4-DCP respectively); large variability in 2,4-DCP concentrations in triclosan sorption controls at day 1 prevented determination of significance of increase in 2,4-DCP concentrations from day 1 to day 6.

In contrast to sorption controls, 2,4-DCP was not detected in active Lemnaceae reactors over 6-d exposure to triclosan. As surface coverage and mass of Lemnaceae were similar in both active Lemnaceae reactors and sorption controls, quantitatively similar contributions to aqueous depletion from physicochemical processes were expected in active Lemnaceae reactors and sorption controls. Absence of 2,4-DCP in active Lemnaceae reactors suggested that Lemnaceae actively removed 2,4-DCP that was produced from photodegradation of triclosan. Previous studies have rigorously documented uptake, metabolism, and sequestration of 2,4-DCP in Lemnaceae systems (Day and Saunders 2004; Pascal-Lorber et al. 2004). Active uptake of 2,4-DCP by Lemnaceae prevented accumulation of 2,4-DCP in active Lemnaceae reactors exposed to triclosan (Figure 6.11). Overall effect of uptake of 2,4-DCP by Lemnaceae on triclosan aqueous depletion was beneficial and likely contributed to slightly greater aqueous depletion of triclosan in active Lemnaceae reactors at 4 – 6 d by decreasing aqueous and sorbed 2,4-DCP concentrations and potentially increasing energetic favorability of triclosan degradation (Figure 6.10).

Results suggest that Lemnaceae plays both active and passive roles in environmental fate of triclosan through uptake of 2,4-DCP and sorption of triclosan. Results presented herein likely under represent the role of active plant uptake in environmental fate of triclosan. A pH of 8 was chosen for the present study to achieve desired initial concentration of $10 \mu\text{M}$; $10 \mu\text{M}$ triclosan precipitated in reactors and controls at pH 7. Previous studies on uptake of 2,4,5-trichlorophenol (2,4,5-TCP) by *L. minor* have indicated pH-dependence (Tront and Saunders 2006). Active uptake of 2,4,5-TCP by *L. minor* increased with increasing fraction of 2,4,5-TCP in the protonated form. Under current experimental conditions, fraction protonated of 2,4-DCP was

approximately 0.39 (as compared to 0.83 at pH 7), indicating that active uptake by plants was adversely impacted by experimental conditions (i.e., pH 8).

2,4-Dichlorophenoxyacetic acid (2,4-D)

Initial experiments to assess aqueous depletion of 2,4-D in Lemnaceae systems indicated potential aqueous depletion of 2,4-D in active Lemnaceae reactors following a delay of 5 d (Figure 6.12). Loss in microbial degradation, sorption, and physicochemical loss controls was statistically negligible. However, in subsequent assessments of active Lemnaceae reactors and microbial degradation controls, concentration of 2,4-D was constant for the first 24 h and then rapidly decreased to non-detectable concentrations by 3 d and 6 d in active Lemnaceae reactors and microbial degradation controls, respectively. No additional peaks were detected in samples from active Lemnaceae reactors or controls. However, delay in aqueous depletion, combined with aqueous depletion in microbial degradation controls, strongly suggested microbial degradation of 2,4-D in Lemnaceae systems. Results are consistent with previous studies that have documented rapid biodegradation of 2,4-D, following a brief lag period, in surface waters (Rubin et al. 1982; Ingerslev and Nyholm 2000). Biodegradation of 2,4-D proceeds through degradation to 2,4-DCP or 4-chlorophenol. As previously indicated in results from triclosan studies (Figures 6.11), detection of aqueous 2,4-DCP in active Lemnaceae reactors or microbial degradation controls was limited due to uptake in active Lemnaceae reactors and sorption of 2,4-DCP in microbial degradation controls. Absence of aqueous 2,4-DCP in active Lemnaceae reactors and microbial degradation controls does not negate microbial degradation of 2,4-D in Lemnaceae systems, but does emphasize limitations of the experimental approach used for these assessments.

Aqueous depletion of 2,4-D in microbial degradation controls was substantially delayed in Trial 1 when compared to Trial 2 (i.e., three versus one day delay, respectively). Additionally, aqueous depletion was observed in active Lemnaceae

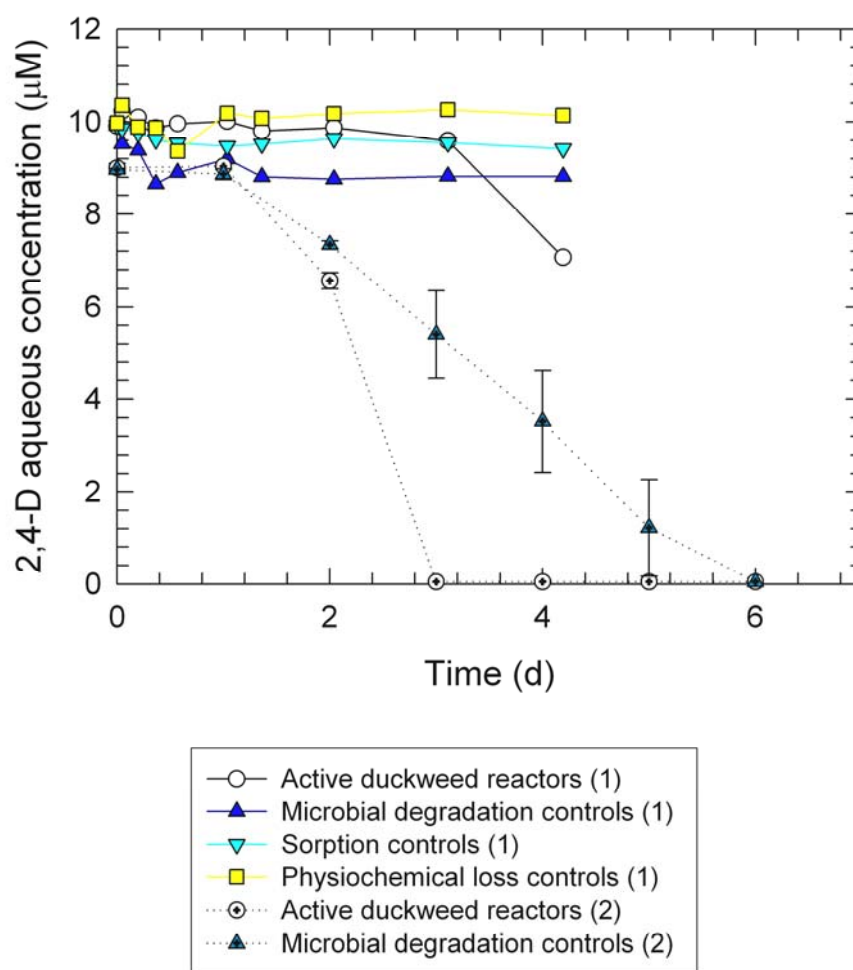


Figure 6.12. Concentrations (μM) of 2,4-dichlorophenoxyacetic acid (2,4-D) in experimental reactors and controls with time (d). Data from Trial 1 (*L. minor*) and Trial 1 (*L. punctata*) are presented. Points represent means and error bars represent standard errors of triplicate reactors.

reactors with *L. punctata* (Trial 2), but was not observed in reactors with *L. minor* (Trial 1). Many potential explanations can account for differences observed in *L. punctata* and *L. minor* assessments, including variation in microbial communities (e.g., numbers and diversity). In experiments utilizing *L. punctata* populations, aqueous depletion was faster in active Lemnaceae reactors than in microbial degradation controls, despite similar delays in aqueous depletion. As lag times prior to aqueous depletion were similar in both active Lemnaceae reactors and microbial degradation, it is unlikely that faster aqueous depletion in active Lemnaceae reactors was attributable to provision of aqueous organic carbon by Lemnaceae to microbial communities that degrade 2,4-D (as was observed in ibuprofen experiments). Uptake of 2,4-DCP, the expected microbial degradation product of 2,4-D, may beneficially affect rates of microbial degradation by decreasing aqueous and sorbed 2,4-DCP concentrations, thus potentially increasing energetic favorability of 2,4-D degradation or decreasing inhibition of microorganisms from 2,4-DCP.

Effects of hydrophobicity on uptake and sorption

Effects of hydrophobicity on aqueous depletion and persistence of organic pollutants in Lemnaceae systems was examined to identify potential trends. Hydrophobicity, measured as octanol-water partitioning coefficient (K_{OW}) has been previously used to predict uptake of organic pollutants in terrestrial systems (Briggs et al. 1982; Burken and Schnoor 1998). However, uptake of halogenated and fluorinated phenols by *L. minor* was not dependent on uptake (Reinhold and Saunders 2006b; Tront et al. 2007), indicating that K_{OW} may have limited predictive value in aquatic plant systems. Uptake of fluoxetine ($\log K_{OW} = 4.05$) by Lemnaceae is inconsistent with $\log K_{OW}$ relationships developed for uptake of organic pollutants by terrestrial plants, as these relationships predict negligible uptake of organic pollutants with $\log K_{OW} > 4.0$. Additionally, ibuprofen and clofibric acid, which possess $\log K_{OW}$ values that bracket that of fluoxetine (e.g., $\log K_{OW} = 3.79$ and 4.66 vs. 4.05), were not uptaken by Lemnaceae,

indicating limited value of K_{OW} in predicting uptake of experimental organic pollutants by Lemnaceae.

Hydrophobicity was also not a reliable predictor of sorption of experimental organic pollutants to Lemnaceae. While most hydrophobic organic pollutants were sorbed to Lemnaceae plants (i.e., fluoxetine and triclosan), sorption of ibuprofen was negligible, even in microbial degradation controls with increased surface area for sorption. However, significant sorption of DEET to macerated Lemnaceae in microbial degradation controls was observed, despite lower hydrophobicity than ibuprofen ($\log K_{OW} = 2.18$ vs. 3.79 , respectively). It is important to note that, with the exception of DEET, provided K_{OW} values are for experimental organic pollutants in ionized forms, as experimental pH conditions favored protonation (i.e., fluoxetine) or deprotonation (i.e., ibuprofen, clofibric acid, triclosan, and 2,4-D). Despite lack of a trend between sorption of experimental organic pollutants to Lemnaceae and hydrophobicity at $\log K_{OW} < 4.0$, results indicate that it can be generalized that sorption to plant tissues will be an important contributor to fate in wetlands for organic pollutants with $\log K_{OW}$ values > 4.0 .

Summary

A summary of results is provided in Table 6.2. Active plant uptake contributed to aqueous depletion of three experimental organic pollutants, both directly (i.e., fluoxetine) and indirectly via uptake of degradates of experimental organic pollutants (i.e., triclosan and 2,4-D). Additionally, passive plant processes, including sorption and enhancement of degradation by plant-associated microorganisms, accounted for aqueous depletion of four experimental organic pollutants (i.e., ibuprofen, fluoxetine, triclosan, 2,4-D).

Fluoxetine and triclosan were most rapidly depleted in active Lemnaceae reactors. Slower aqueous depletion was observed for ibuprofen and 2,4-D, while negligible depletion was observed for DEET and clofibric acid. It is important to emphasize that, as

Table 6.2. Summary of results from active Lemnaceae reactors and controls. Check marks indicate aqueous depletion was observed and aqueous depletion processes observed in this study are listed.

Organic pollutant	Active Lemnaceae reactors	Microbial degradation	Sorption	Physico-chemical loss	Processes
Ibuprofen	✓	✓			Plant-enhanced microbial degradation
Fluoxetine	✓		✓		Sorption and plant uptake
Clofibric acid					None
Triclosan	✓	✓	✓	✓	Photodegradation to 2,4-DCP; uptake of 2,4-DCP
DEET					None
2,4-D	✓	✓			Microbial degradation to 2,4-DCP; uptake of 2,4-DCP

sorption does not contribute to mass removal of organic pollutants, faster aqueous depletion in active Lemnaceae reactors does not necessarily equate to decreased persistence in wetland systems. However, parallel mass removal processes were observed in both fluoxetine and triclosan reactors (i.e., uptake and photodegradation, respectively). Plant uptake accounted for approximately 50% of aqueous depletion of 10 μ M fluoxetine, indicating potential for rapid mass removal of fluoxetine in wetland systems. Additionally, photodegradation contributed to >95% mass removal of 10 μ M triclosan in physicochemical controls. While photodegradation probably accounted for a smaller portion of aqueous depletion in active Lemnaceae reactors because of experimental design to minimize light penetration, results suggest that photodegradation will greatly affect fate of triclosan in wetlands. Consequently, assessments indicated that wetland removal of fluoxetine, triclosan, ibuprofen, and 2,4-D was probable.

Insights gained from assessments of aqueous depletion of organic pollutants in active

Lemnaceae reactors will be valuable in guiding future research of environmental and wetland fate of organic pollutants, including pharmaceuticals, pesticides, and personal care products. For example, active plant uptake of fluoxetine in Lemnaceae systems indicates a need to identify plant metabolic pathways for fluoxetine, and associated enzymes, in aquatic plants. Additionally, research results confirmed that active plant uptake of known products of triclosan and 2,4-D and demonstrated beneficial effects of active uptake of 2,4-DCP on overall aqueous depletion of triclosan and 2,4-D. Consequently, results demonstrated that active plant uptake of degradation products of organic pollutants enhances overall removal of organic pollutants, providing an indirect, but active, role for aquatic plants in fate and removal of organic pollutants in wetlands.

CHAPTER 7: INDUCTION OF LEMNACEAE CALLUS CULTURES

Introduction

Lemnaceae plants, small aquatic plants commonly known as duckweed, are widespread and common free-floating monocotyledonous plants in temperate freshwater environments (Landolt 1986). With rapid growth resulting in dense mats that may contain more than one Lemnaceae species, Lemnaceae reproduce asexually. Due to widespread presence and simple morphology, Lemnaceae plants have been adopted as model organisms for studies on toxicity (ASTM 1997; APHA et al. 1998b) and plant uptake of organic pollutants (Day and Saunders 2004; Reinhold and Saunders 2006b; Tront et al. 2007).

While *in vivo* assessments using Lemnaceae have yielded invaluable knowledge on fates and toxicities of organic pollutants in plant systems, *in vitro* assessments has great potential to advance understanding of phytotoxicity, plant uptake, and phytometabolism in wetlands and surface waters. Use of plant cell suspension cultures to study phytometabolism of organic pollutants is well established for terrestrial plants. For example, cell suspension cultures allowed for rapid and reproducible determination of TCE fate in poplar cells while excluding *in vivo* processes like volatilization and transpiration that complicate elucidation of plant TCE metabolism (Shang and Gordon 2002). Likewise, comparison of plant metabolism of bisphenol A in multiple plant species, including soybean (*Glycine max*), wheat (*Triticum aestivum*), foxglove (*Digitalis purpurea*), and thorn apple (*Datura stramonium*), was facilitated by use of heterotrophic plant cell suspension cultures (Schmidt and Schuphan 2002). Additional studies have examined phytotoxicity of xenobiotic organic pollutants using carrot and soybean cell suspension cultures (Harms 1992; Wang et al. 1996). Unlike these and other contributions of plant cell suspension cultures to terrestrial plant metabolism and toxicity

studies, use of aquatic plant tissue cultures for similar studies is lacking.

Plant tissue culture of Lemnaceae has focused on induction and growth of callus cultures for genetic transformation and plant physiology studies. Callus was first induced in *Lemna gibba* L. using 2,4-dichlorophenoxyacetic acid (2,4-D) and N6-(2-isopentenyl)adenine (2iP) (Chang and Chiu 1976). 2,4-D has also been used, alone and in combination with 6-benzyladenine (BA), 2-iP, and/or dicamba, to induce and maintain callus in *Lemna minor* (Frick and Morley 1995; Stefaniak et al. 2002; Gunter et al. 2004) and *Landoltia punctata* (Li et al. 2004). Three types of callus have been observed in callus culture of *L. gibba*: compact white-yellow callus, friable white callus, and compact green callus that is variable to degree of cellular organization (Chang and Chiu 1976; Moon and Stomp 1997). In-depth comparisons of effects of phytohormones, basal nutrient medium, carbohydrates, and light have been conducted previously for a variety of Lemnaceae species (Moon and Stomp 1997; Li et al. 2004). Most callus induction methods were developed for Lemnaceae clones that have been maintained in laboratory conditions for extended periods of time (e.g., 10 to 40 yr); however, callus induction of *L. minor* obtained from natural sources in Russia has been reported (Gunter et al. 2004). Additionally, with the intents of genetic transformation or plant physiology studies, the above studies do not report development of Lemnaceae cell suspension cultures.

As callus cultures can differ significantly based on varietal origin, the primary objective of this study was to induce and maintain callus cultures from Lemnaceae species collected from a tertiary treatment wetlands used to polish municipal wastewater effluents. Callus induction studies evaluated six published methods for callus induction success in *Lemna minor* and *Landoltia punctata* collected from treatment wetlands. Subsequent research aimed to assess the suitability of formed callus for initiating cell suspension cultures.

Materials and Methods

Basal nutrient media and phytohormones

Stock solutions for basal nutrient media were created from salts, organics, and vitamins provided by Fisher Scientific (Pittsburg, PA), Sigma-Aldrich (St. Louis, MO), and VWR (West Chester, PA). Basal nutrient media for callus induction included Murashige and Skoog (MS) medium (Murashige and Skoog 1962), Gamborg (B5) medium (Gamborg et al. 1976), and McCown woody plant (WP) medium (Lloyd and McCown 1980). Inorganic and trace nutrient stocks were stored at 4°C, while organic stocks were stored at approximately -15°C. Phytagel (Sigma-Aldrich, St. Louis, MO) was used as a solidifying agent (3 g/L) for callus induction and maintenance medium. Medium was autoclaved at 120°C and 1.2 atm for 20 to 30 min, depending on volume of medium being sterilized.

Phytohormones were added prior to autoclaving of callus induction and maintenance medium. Measurements of phytohormone concentrations prior to, and after, autoclaving indicated negligible change in concentration of phytohormones, minimizing the need for addition of filter-sterilized phytohormones after autoclaving. Phytohormones, abbreviations, source, and solvents used to create phytohormone stocks are provided in Table 7.1.

Collection of Lemnaceae species

Lemnaceae species of the Lemnoideae subfamily were collected from a constructed wastewater effluent treatment wetland operated by Clayton County Water Authority (CCWA; Jonesboro, GA). Species were identified using 10 to 40X light and phase contrast microscopy using an internet dichotomous key (Armstrong 2001). Species identification indicated that *Lemna minor* and *Landoltia punctata* were dominant Lemnaceae species in the treatment wetland; however, *Spirodela polyrrhiza* was also

Table 7.1. Phyto regulators used for callus induction and maintenance studies with chemical suppliers and solvents used for stock solutions. Phyto regulators were supplied by Alfa Aesar (AA; Heysham Lancs.), Research Organics (RO; Cleveland, OH), Chem Service (CS; West Chester, PA), and Pfaltz and Bauer, Inc. (PB; Waterbury, CT).

Phyto regulator	Supplier	Solvent for stock
2,4-dichlorophenoxy-acetic acid (2,4-D)	AA	0.1 N NaOH
<i>N</i> ⁶ -(3-methylbut-2-enyl)adenine (2-iP)	RO	1 N NaOH
6-benzylaminopurine (BA)	AA	1 N NaOH
1-napthalene acetic acid (NAA)	RO	1 N NaOH
Thiadiazuron (TDZ)	PB	DMSO
Dicamba	CS	Ethanol

occasionally present as a member of Lemnaceae communities dominated by *L. minor* or *L. punctata*. *Wolffia* species, members of the Wolffioideae subfamily of Lemnaceae, were also occasionally present as members of Lemnaceae communities and were separated from species of the Lemnoideae subfamily using a No. 10 U.S.A. Standard testing sieve with 2.00 mm openings. Prior to disinfection, Lemnaceae communities were maintained in Standard Methods medium (APHA et al. 1998b) under a 16-8 h photoperiod.

Disinfection of Lemnaceae species

Lemnaceae species were rinsed with distilled water for one hour and debris and insects were removed. Development of disinfection procedures compared five methods of disinfection for *L. punctata*. For each method, approximately 30 to 50 *L. punctata* fronds were placed in small mesh tea balls for ease of immersion, mixing, and transfer of fronds. Fronds were immersed and agitated in (a) 1.2% sodium hypochlorite for 4 min, (b) 1.2% sodium hypochlorite for 6 min, (c) 0.6% sodium hypochlorite for 10 min, (d) 0.6% sodium hypochlorite for 20 min, or (e) 10% hydrogen peroxide for 10 min. After immersion in disinfecting agent, fronds were immersed and agitated for 1 min in three consecutive rinses of sterile nanopure water. To immediately test efficiency of

disinfection, fronds were placed on Murashige and Skoog medium (MS) and plates were visually monitored for growth of microbial contaminants. Additionally, individual fronds from disinfected frond lines were inoculated in sterility test medium that was subsequently monitored for microbial growth for 7 d (Leifert et al. 1989). Twenty fronds from each treatment were plated on MS medium and monitored for contamination and growth. Groups of non-contaminated *L. punctata* fronds were transferred to new MS medium every three to four weeks. Each grouping of fronds was considered genetically similar since *L. punctata* vegetatively reproduces. Reproduction rates of resulting frond lines were measured by counting frond number per grouping after transfer of single fronds to new medium.

Based on results from disinfection studies, fronds for callus induction were disinfected in 0.6% hypochlorite until edges of the fronds turned white. Small mesh tea balls were not used to contain the fronds, as their ability to contain fronds was quickly compromised by corrosion. After three subsequent rinses in sterile nanopure water, fronds were grown on solid Hoagland's medium with 3% sucrose for two to four weeks to monitor for microbial contamination. Non-contaminated, axenic fronds were maintained under constant light in liquid Hoagland's medium with 0.1% to 2% sucrose, depending on desired frond reproduction rates.

Callus induction methods

Six methods were assessed for successful callus induction from Lemnaceae collected from CCWA wetlands. Four published methods for callus induction in Lemnaceae were selected for *L. minor* studies: the C method (Chang and Chiu 1976), G method (Gunter et al. 2004), S method (Stefaniak et al. 2002), and F method (Frick and Morley 1995), while two published methods for callus induction in Lemnaceae were selected for *L. punctata* studies: the M method (Moon and Stomp 1997) and L method (Li et al. 2004). Methods were selected to cover a broad range of culture conditions, basal

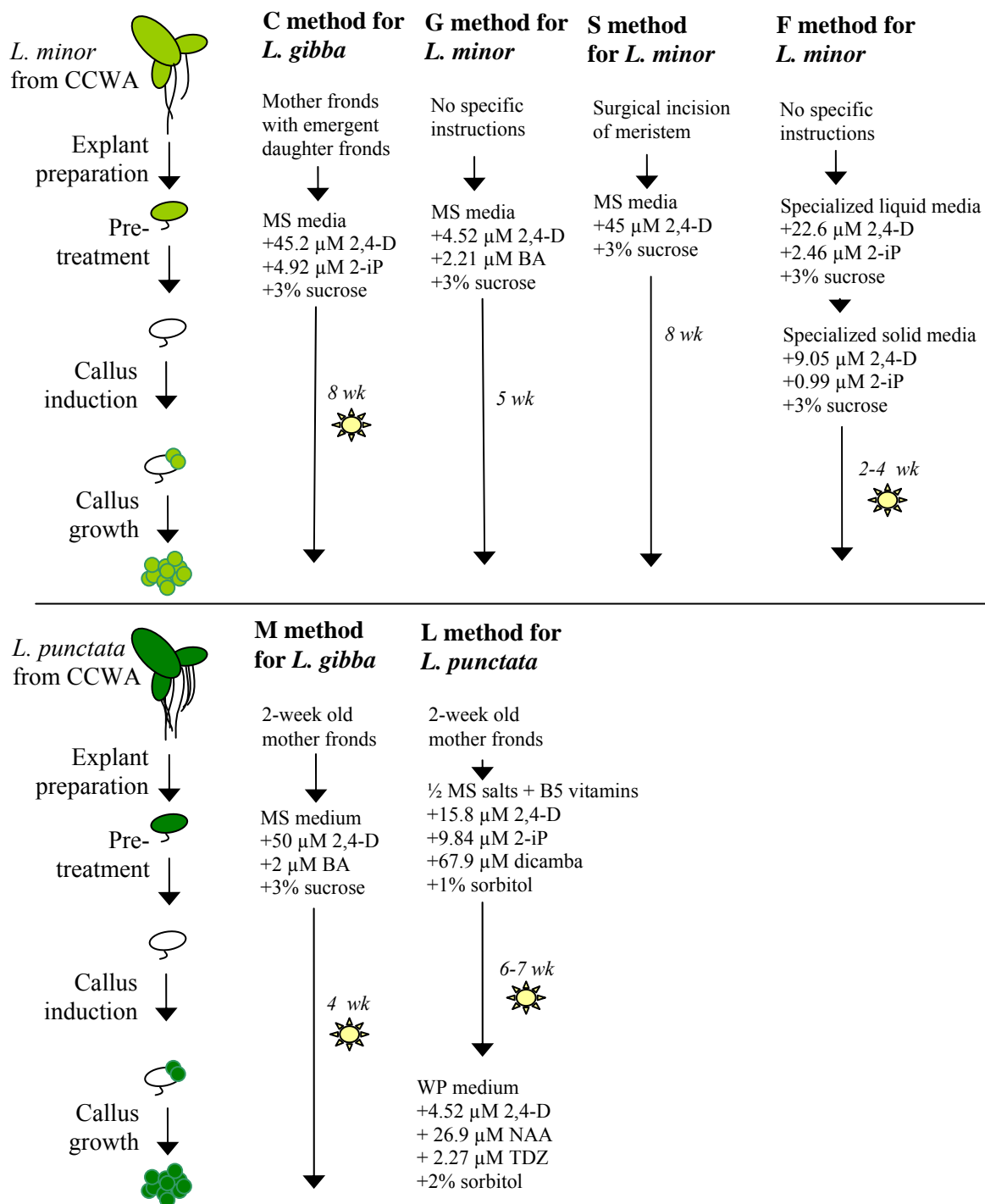


Figure 7.1. Outline of callus induction methods evaluated for *L. minor* and *L. punctata* from CCWA wetlands. Lemnaceae species used for original development of callus induction methods by Chang and Chiu, 1976 (C); Stefaniak et al., 2002 (S); Gunter et al., 2004 (G); Frick and Morley, 2005 (F); Moon and Stomp, 1997 (M); and Li et al., 2004 (L) are provided.. Sun represents partial or continuous light exposure during induction and growth. Abbreviations of phytohormones are provided in Table 7.1.

growth medium, and phytohormones, and were not necessarily developed for use with *L. minor* or *L. punctata*. A summary of these methods, with identification of species which method was initially intended, is depicted in Figure 7.1. A minimum of 500 explants were prepared for each method. Additionally, a minimum of three frond lines were used for each method to increase the genotypic and phenotypic variability of explants.

Procedures for the C method for *L. minor* closely followed a method developed previously for *L. gibba* (Chang and Chiu 1976), with the exception that 60 x 15 mm Petri dishes were used instead of 125-mL culture flasks. Mother fronds, with emergent daughter fronds, were plated after removal of daughter fronds. Seven axenic fronds were plated adaxial side down on each plate containing MS medium, 45.2 μ M 2,4-D, 4.92 μ M 2-iP, and 3% sucrose. For the C method, and all other methods, 3 g/L Phytigel (Sigma, St. Louis, MO) was used as a solidifying agent. Plates were maintained at 25°C under a 12:12 light: dark photoperiod with light provided by a combination of fluorescent and plant growth lights. Callus induction frequency was assessed after 8 wk of culture. Callus cultures were subsequently transferred every four to six wk to 100 x 15 mm Petri dishes, when sufficient growth was observed and prior to differentiation and/or organization of callus cells.

The G method was adapted from procedures previously published for callus induction for *L. minor* collected from natural sources in Russia (Gunter et al. 2004). Prior to plating, mother fronds were separated from daughter fronds and only fully developed fronds were selected for plating. Approximately 30 axenic fronds were placed adaxial side down on 100 x 15 mm Petri dishes containing solidified MS medium with 4.52 μ M 2,4-D, 2.21 μ M BA, and 3% sucrose. Plates were maintained at 22°C without exposure to light. Frond growth and callus induction frequency were measured after 5 wk.

The S method for *L. minor* callus induction differed from other methods in that fronds were surgically incised in the meristematic region prior to plating, as previous

results indicated increase in induction frequency from incisions in explants (Stefaniak et al. 2002). Fully-developed fronds were gently incised from the center to the base. Roots and daughter fronds, if present, were generally removed when fronds were incised. Care was taken to incise only the abaxial surface of the fronds and not to incise completely through fronds. Successfully incised fronds were plated on solidified MS medium with 45 μ M 2,4-D and 3% sucrose. Approximately 20 fronds were placed on each 100 x 15 mm plate. Plates were maintained at 25°C in complete darkness for 8 wk prior to assessment of callus induction frequency.

The F method was the most complicated of all methods attempted for *L. minor* callus induction. Based on methods developed for a *L. minor* L. clone that had been maintained under laboratory conditions for over 20 years (Frick 1991; Frick and Morley 1995), F method utilized a preconditioning period followed by growth on solidified medium slants. Additionally, F method used a basal nutrient medium specifically developed for callus induction in *L. minor* L. (Frick 1991). Axenic fronds were first grown in specialized liquid medium containing 22.6 μ M 2,4-D, 2.46 μ M 2-iP, and 3% sucrose. After 7 d, cultures (consisting of fronds and beginning formations of callus) were scraped through a stainless steel screen with approximately 1 mm openings to separate frond and root tissue from any callus. Tissues that passed through the stainless steel screen were plated on solidified nutrient medium slants containing 9.05 μ M 2,4-D, 0.99 μ M 2-iP, and 3% sucrose. Slants were maintained under continuous light at 25°C and were assessed for callus induction frequency after 6 – 8 wk.

Initial attempts to induce callus in *L. punctata* utilized procedures initially developed for *L. gibba* [i.e., M method adopted from Moon and Stomp (1997)]. Two-wk-old mother fronds were separated from daughter fronds and placed adaxial side down on solidified MS medium with 50 μ M 2,4-D, 2 μ M BA, and 3% sucrose. Roots, if present, were shortened but not removed. Approximately 10 or 30 fronds were plated on each 60 x 15 mm or 100 x 15 mm Petri dish, respectively. Plates were maintained under

a 16:8 hr light: dark photoperiod. Callus induction frequency was measured after 4 wk. Based on previous studies (Moon and Stomp 1997; Stomp and Rajbhandari 2000; Yamamoto et al. 2001), a modification of the M method was also assessed for callus induction frequency. Explants were treated as previously described for the M method, with the exception that phytohormone concentrations included combinations of 2.5, 5.0, and 7.5 μM 2,4-D and 1.0 and 2.0 μM BA.

The second method for *L. punctata* callus induction (i.e., L method) was developed previously for induction of *L. punctata* 8717 callus suitable for regeneration (Li et al. 2004). L method utilized a two-step process for callus induction and growth. Mature mother fronds (2 – 5 d old) were initially plated (adaxial side down) on solidified medium consisting of $\frac{1}{2}$ MS salts, B5 vitamins, phytohormones, and sorbitol for a pretreatment period of 6 – 7 wk. Three phytohormones, i.e., 15.8 μM 2,4-D, 9.84 μM 2-IP, and 67.9 μM dicamba, were used in pretreatment medium. Sorbitol was selected as a carbon source for pre-treatment and subsequent callus induction to decrease frond growth prior to callus induction (Li et al. 2004). After 2 wk of pretreatment, mother fronds that had produced daughter fronds were transferred to fresh pretreatment medium and senescent fronds were discarded. After 6 – 7 wk, callus was transferred to callus growth medium containing WP nutrients, 4.52 μM 2,3-D, 26.9 μM NAA, 2.27 μM TDZ, and 2% sorbitol and callus induction frequency was assessed.

Assessment of callus induction frequency

Callus induction frequency was measured using phase-contrast microscopy and light microscopy at 10 to 40 X magnification. Swelling of fronds, spikes or bumps on the surface of fronds, and extreme deformation of fronds prior to measurement of callus induction frequency were noted, as previous studies may have counted similar tissues as callus (Moon and Stomp 1997). Tissue was counted as callus if growth was unorganized

or undifferentiated, friable, and green, white, or yellow. If uncertainty about nature of growth existed, the tissue was transferred to new medium and monitored for growth. If subsequent increase in callus-like tissue was observed within 3 - 4, the tissue was counted as callus. Senescent fronds or partially undifferentiated tissues that were black, brown, or red in color were not counted as callus.

Callus induction frequency was calculated by dividing total number of fronds producing callus by total number of fronds plated for each method. Plates that were discarded due to presence of microbial contamination were excluded from callus induction frequency calculations. While this calculation did not account for production of multiple callus from one frond, this method of calculation was selected because previous use by Li et al. (2004), Chang and Chiu (1976), and Stefaniak et al. (2002) allowed for comparison of callus induction frequency with previous studies. Callus induction frequency for the C method was estimated, as the hard, friable, and round nature of C callus complicated callus counts; in other words, separation and movement of C callus on solidified medium may have led to artificially high callus counts.

Initiation of cell suspension from callus cultures

Based on robust callus growth, callus from the L and C methods were chosen for cell suspension initiation studies. Approximately 1 to 2 g of callus was inoculated into 50 mL of liquid callus maintenance medium in 125-mL culture flasks. Inoculums consisted of 3 to 5 mm callus that had been scraped through 1-mm stainless steel screens, creating inoculums with greater surface areas for subsequent cellular growth. Inoculated medium was kept under constant light at room temperature (22 to 24°C) on orbital shakers. Success of cell suspension initiation was measured over four weeks by monitoring increase in density of cells in liquid medium and visual observations of medium at 40 to

100X magnification.

Results and Discussion

Disinfection of Lemnaceae species and subsequent growth of frond lines

Bleach and hydrogen peroxide were both effective disinfecting agents for *L. punctata*. As shown in Figure 7.2, non-contaminated, healthy fronds were achieved in all treatments, excluding the treatment of 1.2% sodium hypochlorite for six minutes. More microbial contamination was observed when lower (e.g., 0.6%) concentrations of sodium hypochlorite or 10% hydrogen peroxide were used as disinfecting agents. However, use of 1.2% sodium hypochlorite was complicated by the short period of time between effective bleaching, as indicated by beginning of chlorosis or whitening of edges of fronds, and excessive bleaching, as indicated by complete chlorosis or complete whitening of fronds. The absence of growth when fronds were treated with 1.2% sodium hypochlorite for 6 min resulted from excessive bleaching. As transferring fronds from the disinfection agent to the first rinse of sterile water frequently required more than 30 sec, the short period of effective, but not excessive, bleaching for 1.2% sodium hypochlorite limited its practicality as an effective method for disinfection of *L. punctata* fronds. Growth of *L. punctata* fronds was similar for both 0.6% sodium hypochlorite and hydrogen peroxide, indicating that both disinfection agents could be used for successful disinfection of fronds. 0.6% sodium hypochlorite was chosen as the preferred disinfecting agent for subsequent studies.

Six lines of axenic, healthy fronds were selected for determination of frond reproduction rates. Growth of *L. punctata* fronds on solid MS medium was generally rapid, with an average doubling time of 2.90 ± 0.52 d. As shown in Figure 7.3, number of *L. punctata* fronds after 9 d of growth on MS medium ranged from 4.9 ± 2.8 fronds (plate A) to 9.0 ± 1.2 fronds (plate E). Rapid reproduction rates of axenic fronds allowed

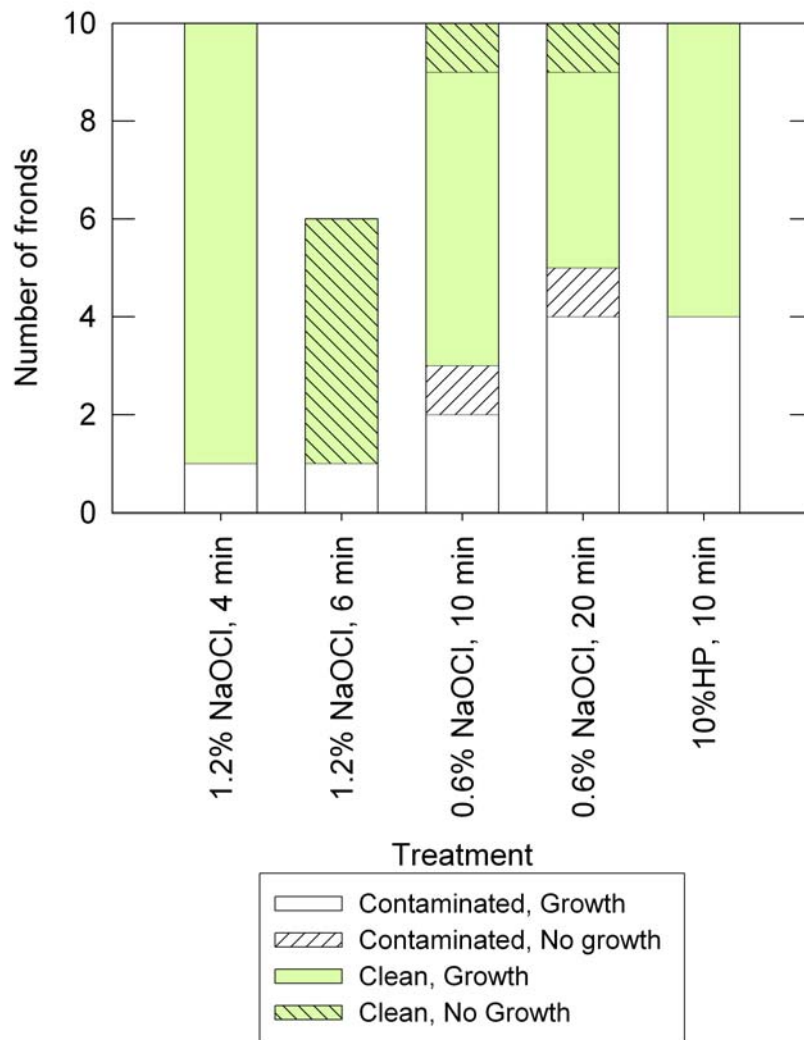


Figure 7.2. Growth and contamination of fronds after disinfection treatments. NaOCl represents sodium hypochlorite and HP represents hydrogen peroxide.

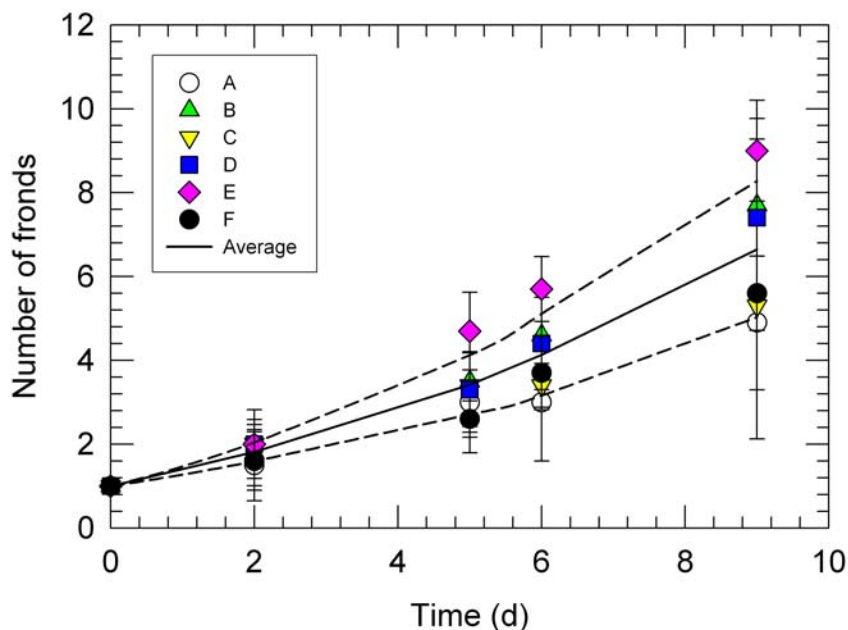


Figure 7.3. Growth of axenic fronds on MS medium. Solid line represents average and dashed lines represent standard deviation of all frond colonies (n=6).

for culture of genotypically-similar fronds for callus induction, but necessitated frequent transfer approximately weekly. After initial frond reproduction measurements, fronds were transferred to liquid Hoagland's medium with 0.1% to 2.0% sucrose, depending on desired reproduction rates and transfer frequency.

Callus induction in *L. minor* and *L. punctata*

Of the six callus induction methods attempted, three methods successfully induced callus in Lemnaceae collected from CCWA tertiary treatment wetlands. Callus induction for the C and S methods for *L. minor* were successful, with induction frequencies of >99% and 35.2%, respectively. For *L. punctata*, the L method was successful, with induction frequency of 75.6%. F, G, and M methods yielded few or no callus cultures and were classified as unsuccessful. Table 7.2 briefly summarizes observations and callus induction frequencies for the six experimental methods.

Table 7.2. Summary of callus induction frequency and brief observations of callus growth for experimental methods.

Method	Species	Observations	Callus induction frequency
C	<i>L. minor</i>	Combination of hard green and crumbly white/yellow callus	>99%
G	<i>L. minor</i>	Senescent fronds with elongated stipules	0%
S	<i>L. minor</i>	Yellow callus characterized by soft tissue growing on surface of daughter frond and clusters of small, hard amorphous masses	35.2%
F	<i>L. minor</i>	Senescent, organized tissue and small green callus-like tissue that did not grow after transfer	0%
M	<i>L. punctata</i>	Few senescent frond with small round domes of tissue in meristematic region that did not grow after transfer	0%
L	<i>L. punctata</i>	Dark and light green, hard, crumbly callus, with some partially differentiated tissues	75.6%

C method was the most effective method for callus induction, with an estimated induction frequency of >99%. After three to four wk of exposure to callus induction medium, *L. minor* fronds had curled up or deformed and spikes were visible on surface of fronds at 20 – 40 X magnification. Plated mother fronds produced daughter fronds prior to callus induction and callus induction was observed primarily on daughter fronds. After 8 wks of culture, majority of mother fronds were senescent, as indicated by pale brown or yellow frond coloration. Daughter fronds had morphed into hard, spherical amorphous masses that subsequently became callus. The nature of these masses complicated association of callus with its parent explant, as the amorphous masses were not stationary on the plates; i.e., it was difficult to determine the number of callus produced per explant and the high induction frequency represents total numbers of callus and explants. At the

beginning of callus induction, callus was typically dark green, but white, yellow, and light green callus were observed after subsequent transfers. White and yellow callus was rarely observed at 7 wk of culture, but increased in quantity and ratio with time in culture. After 6 mo of culture, with monthly transfers to new medium, callus consisted of approximately equal quantities of white, yellow, and green callus. Examples of callus at the beginning of callus formation (approximately 8-9 wk) and after long-term culture (greater than 6 mo) are provided in Figure 7.4. Partial differentiation of callus, leading to frond-like tissues, occurred if callus was not transferred more frequently than every 6 wk. Consequently, reselection of callus was required to maintain healthy callus cultures. Previous studies have also cited the necessity of reselection to callus maintenance (Moon and Stomp 1997), indicating that some types of callus may naturally partially differentiate and cease growth on callus induction medium.

The G method was not successful at inducing callus for *L. minor* collected from CCWA wetlands. When assessed after 5 wk of culture in complete darkness, each explant had produced two to three additional daughter fronds. Curling of daughter fronds away from medium was frequently observed. Additionally, stipules were elongated, allowing for separation of mother and daughter fronds by approximately 2 – 3 mm. However, no amorphous masses or callus growths were observed and all fronds were senescent, as indicated by pale brown coloration, after 5 wk of culture. Therefore, the G method was deemed unsuccessful for callus induction in *L. minor* collected from CCWA wetlands.

The second method that cultured explants and callus in the dark was the S method. The S method differed from the G method in that fronds were surgically incised prior to plating, concentration of 2,4-D was 10 times greater, and no BA was added to culture medium. Additionally, culture time prior to assessment of callus induction was 8 wk instead of 5 wk. Callus induction frequency in the S method was 35.2%, indicating that one, or a combination, of these changes in procedure yielded successful callus

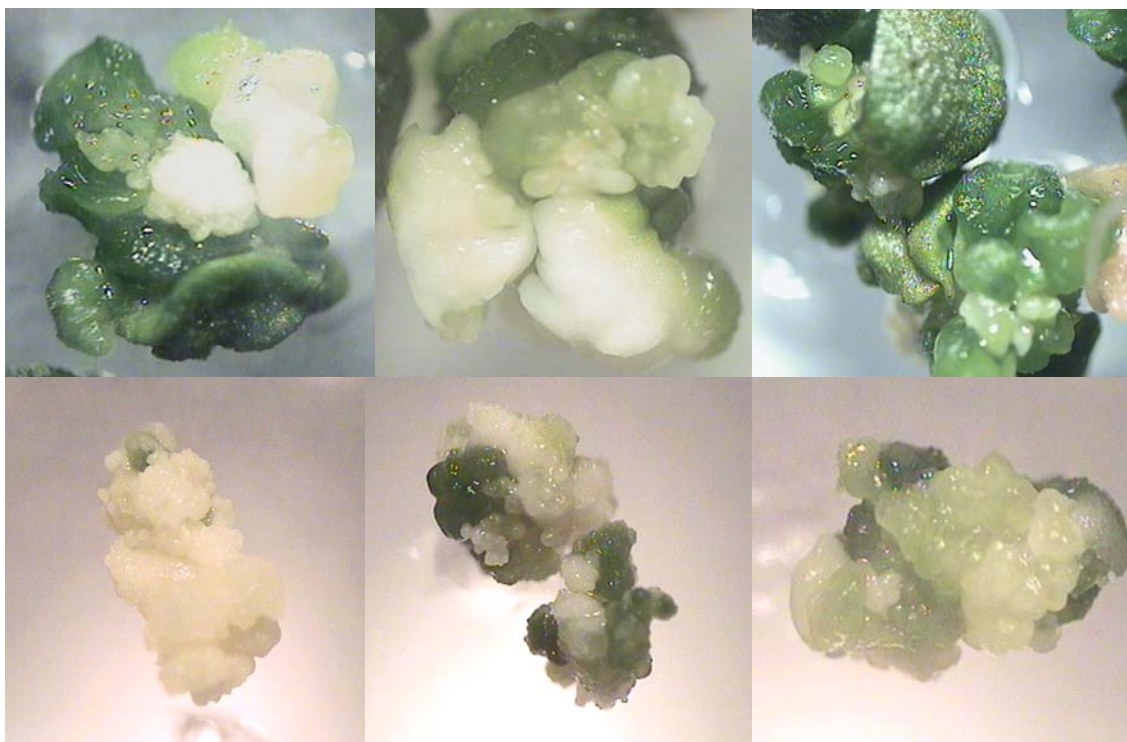


Figure 7.4. *L. minor* callus induced and maintained using the C method adopted from Chang and Chiu (1976). Top three photos are of explants in early stages of callus culture (approximately 8 to 9 wk). Note presence of differentiated frond-like tissues. Bottom three photos are of callus after long-term (greater than 6 months) culture and are at 10X magnification. Presence of differentiated tissue is reduced and cultures consist of combinations of green, white, and yellow callus.

induction. As with the G method, mother fronds senesced within five to six weeks. However, the size of daughter fronds produced in culture on S medium was greatly reduced. Daughter fronds were small (< 3 mm), round, yellow, and occasionally swollen. Similar to the G method, daughter fronds curled away from S medium after five weeks in culture. Callus typically originated from daughter fronds, although some callus originated from the incision of the mother frond, as shown in Figure 7.5. Callus was initially yellow, although white callus formed on yellow callus after multiple transfers and six to seven months in culture. Callus was slow growing and difficult to maintain; many cultures only reached early stages of callus development and died after few months in culture. Callus induction frequency in this study was substantially less than previously



Figure 7.5. *L. minor* callus induced with the S method based on Stefaniak et al. (2002). Photographs show the range of callus formation, from callus originated from daughter fronds and meristematic incision (left and center photographs, respectively) to fully developed friable callus (right photograph).

reported callus induction efficiency of approximately 89% (Stefaniak et al. 2002).

The fourth method (i.e., F method) attempted for callus induction in *L. minor* collected from CCWA wetlands=was largely unsuccessful. After pretreatment of fronds in liquid medium, a few small, hard amorphous masses were observed on fronds after 2 wk. However, mechanical separation of explants by scraping through stainless steel screen did not efficiently separate hard amorphous masses from small fragments of frond tissues. Consequently, pre-formations of callus and differentiated tissues were both plated on callus induction slants. Neither pre-formations of callus nor differentiated tissues produced sustainable callus in the expected 2 – 4 wk callus induction and growth period. Therefore, the F method was considered unsuccessful for callus induction in *L. minor* collected from CCWA tertiary treatment wetlands.

Both successful methods for *L. minor* callus induction utilized approximately 45 μM 2,4-D concentrations, whereas unsuccessful methods utilized 4.52 μM or 22.6 μM 2,4-D, indicating that high concentrations of 2,4-D were required for callus induction in *L. minor* collected from CCWA tertiary treatment wetlands. Additionally, callus was successfully induced in complete darkness. Previous studies have reached inconsistent

conclusions about the ability of grow Lemnaceae callus in the dark. Chang and Chiu (1976) reported formation of slow growing white callus of *L. gibba*; however, after observing inability to reproduce *L. gibba* callus growth in the dark, Moon and Stomp (1997) have conjectured that dark conditions created by Chang and Chiu (1976) did not completely prevent incidental light exposure to callus. Consequently, extreme care was taken during this study to prevent incidental light exposure to S method callus, including inclusion of cultures in a dark box, placement of dark box above any lights in the climate control room, and infrequent assessments of cultures. Therefore, this study demonstrated growth of *L. minor* callus in the dark, repeating previously published results (Stefaniak et al. 2002).

The method developed by Moon and Stomp (1997) for callus induction in *L. gibba* was adapted in this study as the M method for *L. punctata*. After 8 wk of culture on callus induction medium, fronds were typically light yellow or brown and normally shaped. Fronds had reproduced prior to senescence and green, white, or purple fronds were observed. Partial dedifferentiation was infrequently observed. Partially undifferentiated pieces were transferred to new medium after four to six weeks. Growth of callus from these partially undifferentiated pieces was not observed. Adjusting concentration of BA (1 – 2 μ M) or 2,4-D (2.5 – 7.5 μ M) did not induce callus. It is important to note that Moon and Stomp (1997) report a callus induction frequency of only 10% for *L. gibba*. Therefore, inability to induce callus using the M method for *L. minor* may have resulted from the specificity of the method from *L. gibba* combined with low induction frequency even with the intended species of Lemnaceae.

L. punctata callus was successfully induced utilizing the method previously developed by Li et al. (2004). Within the first two weeks of culture on the pre-treatment medium, over 80% of explants had produced daughter fronds. Daughter fronds curled away from the medium and spikes were observed on the surfaces of the fronds. Green callus was observed prior to the 7-wk assessment time. Initially only dark green callus

was observed; however, after 5 – 6 mo of culture, pale green callus formed from some of the dark green callus. Pale green callus grew rapidly and required transfer every two weeks, as compared to every three to four weeks for dark green callus. Photographs of both types of callus are shown in Figure 7.6.

Callus induction frequency of 75.6% was slightly less in this study than the previously reported callus induction frequency of 92% (Li et al. 2004). Reduced callus induction frequency may be due to increased genotypic and phenotypic variability of *L. punctata* collected from CCWA tertiary treatment wetlands. Additionally, laboratory culture of axenic fronds may select for *L. punctata* fronds with increased growth under laboratory conditions and increased suitability of fronds for callus induction. As compared with previous studies, this study utilized *L. punctata* fronds that originated from tertiary treatment wetlands and that were maintained under laboratory conditions for the minimal time for axenic growth (i.e., less than two months and typically two weeks). As previously reported (Li et al. 2004), differentiation of *L. punctata* callus was frequently observed in L method cultures. Differentiated tissues typically consisted root-like or frond-like tissues and were manually separated from callus prior to transfer to new medium. Reducing the length of time in between transfers from four weeks to three weeks greatly reduced the number of differentiated tissues, indicating that phytohormone concentrations may have limited callus dedifferentiation after three weeks.

Previous studies have reported multiple types of callus formation from *L. gibba* (Chang and Chiu 1976; Moon and Stomp 1997). All three types of callus, compact, white-yellow callus, friable white callus, and compact green callus that was variable to degree of cellular organization, were observed in C method cultures of *L. minor*. However, only green callus was observed in culture of *L. punctata* in this study and previously (Li et al. 2004). Interestingly, whereas Li et al. (2004) only reports formation of compact dark green callus that was prone to differentiation, this study demonstrated formation of two types of callus – the *L. punctata* callus previously observed by Li et al.

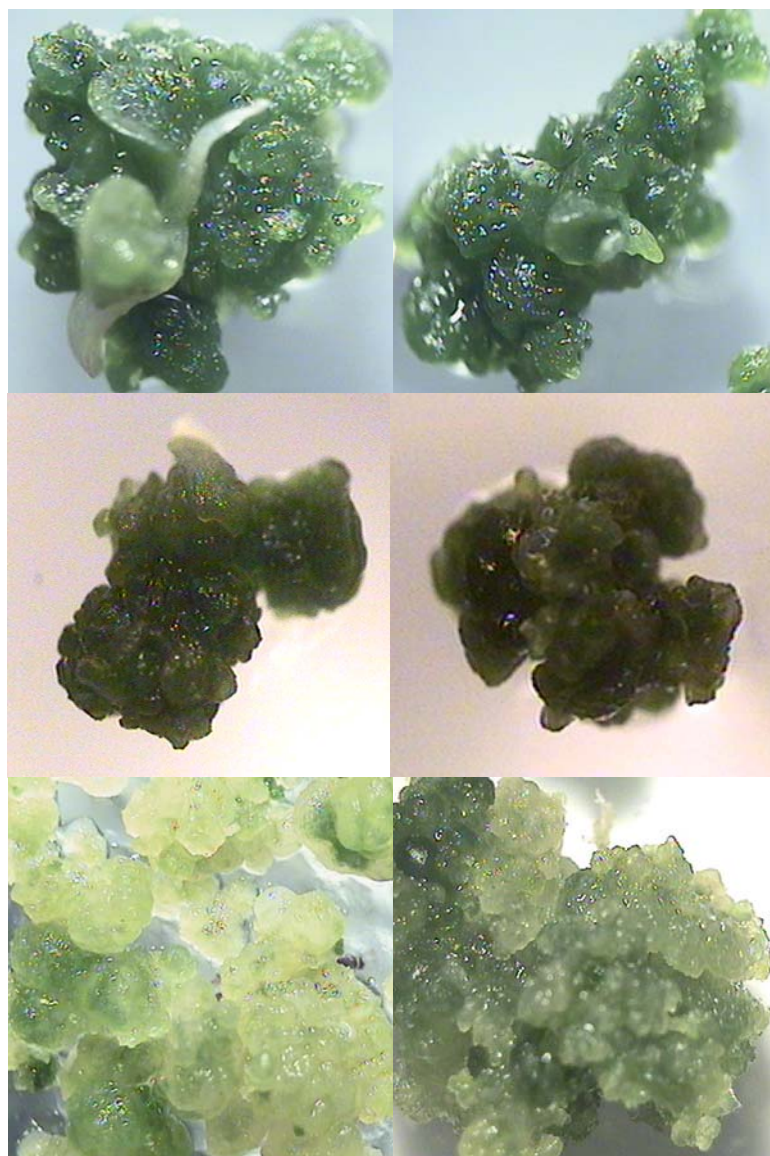


Figure 7.6. *L. punctata* callus induced utilizing method developed by Li, et al. (2004). Photographs were taken after 3.5 months (top row) and 6 months (middle and bottom rows). Two types of callus, dark green and pale green callus, are shown in the middle and bottom rows, respectively.

(2004) and non-compact, pale green callus that was not previously reported. Frequency of non-compact, pale green callus was greater after 6 mo of culture than after 1 mo of culture, indicating that prolonged exposure to callus growth medium promoted formation of non-compact, pale green *L. punctata* callus.

Initiation of cell suspension cultures

Attempts to initiate cell suspension cultures from callus were unsuccessful. Initial attempts, utilizing pale green, fast growing callus from *L. punctata* (i.e., L method callus) that was scraped through 1 mm stainless steel screen, yielded immediately cloudy medium. Cloudiness did not increase over the next two weeks and plant cells were not observed under 40 to 100X magnification, indicating that cloudiness resulted from scrapings of initial plant callus. Transfer of 5 mL of settled plant tissues to new liquid medium verified minimal growth of tissues in suspension, as tissue density in medium did not increase over four weeks. Subsequent attempts to inoculate cell suspensions utilized two to five mm diameter callus pieces to minimize initial cloudiness of inoculated medium. *L. punctata* callus remained green for approximately two weeks, with minimal increase in medium cloudiness, prior to senescence. Negligible quantities of plant cells were observed under magnification. Likewise, increase in medium cloudiness or growth of plant cells in suspension was not observed when liquid C method medium was inoculated with *L. minor* callus.

Summary

This research provides a comparative look at the applicability and efficiency of callus induction methods for Lemnaceae collected from tertiary wastewater treatment wetlands. Three of six methods were successful at inducing callus in *L. minor* and *L. punctata*. Identification of Lemnaceae was crucial to effective callus induction, and results indicate that correct species identification was important in two of three successful

callus induction methods (i.e., S and L methods). The third successful method induced callus in *L. minor* using a method developed initially for *L. gibba* (i.e., C method). However, *L. punctata* callus was not induced using a similar method also developed for *L. gibba* (i.e., M method). As *L. minor* is considered more phenotypically and genotypically similar to *L. gibba* than is *L. punctata* (Les et al. 2002), this study indicates that, if methods for species-specific callus induction methods are not available, phylogenetic taxonomy may yield valuable information for method selection.

Additionally, results from this study further support previous research that has documented callus induction in complete darkness (Chang and Chiu 1976; Stefaniak et al. 2002). However, callus induction frequency was substantially lower under dark conditions and only one type of callus was observed. In contrast, multiple types of callus formed from *L. minor* (three types) and *L. punctata* (two types) when cultures were maintained under continuous or intermittent light, indicating that light energy increases the diversity of callus. The presence of cytokinins in callus induction medium may also contribute to increased callus diversity, as the C method and L method utilized cytokinins (e.g., 2-iP, dicamba, TDZ) in combination with 2,4-D and the S method used 2,4-D alone to induce callus. A new type of Lemnaceae callus was observed in culture of *L. punctata* callus – a non-compact, pale green callus with relatively rapid growth rates that may be well suited for callus toxicity assessments.

Most importantly, this study demonstrated callus induction in Lemnaceae species collected from tertiary treatment wetlands. Culture of fronds prior to callus induction was minimized to increase genotypic similarity between callus cultures and wetland Lemnaceae. Simple bleaching procedures were effective at disinfecting Lemnaceae fronds and growth of axenic fronds under laboratory conditions was rapid. Callus induction typically required eight weeks, and greater than five to six months of culture was required for development of robust and diverse callus with minimal differentiation of tissues. Callus was not suited for development of cell suspension cultures, indicating that

additional studies focusing on development of Lemnaceae cell suspension cultures are needed.

CHAPTER 8: TOXICITY AND METABOLISM IN LEMNACEAE AND LEMNACEAE CALLUS CULTURES

Introduction

Plant tissue cultures hold great promise for advancing understanding of plant interactions with pollutants by facilitating examination of phytotoxicity and phytometabolism at a cellular level. However, previous research has yielded mixed results as to applicability of results from tissue cultures to whole plant systems. Barley, tomato, and carrot cell suspension cultures exhibited species specific toxicities to 4-chloroaniline, 3,4-dichloroaniline, phenanthrene, and 4-nonylphenol (Harms 1992); however, studies have also indicated that plants do not necessarily exhibit traits, including herbicide resistance, that are present in the plant tissue cultures from which the plants were regenerated (Loh 1992). Plant cell suspensions are also useful for studying phytometabolism of many organic pollutants, including pesticides (Sandermann et al. 1984), industrial chemicals like polychlorinated biphenyls (Harms 1996), and explosives like trinitrotoluene and cyclotrimethylenetrinitramine (Mezzari et al. 2004). Phytometabolism studies utilizing plant cell suspension cultures have also yielded mixed results on applicability of metabolism in plant cell suspension cultures to metabolism in whole plants. Wheat and tomato exhibited species-specific metabolism, as classified by polar and nonpolar metabolites and non-extractable residues, of multiple sludge-derived organic pollutants (Harms 1996). Metabolism of chlorinated pesticides (e.g., 2,4-dichlorophenoxyacetic acid, pentachlorophenol, DDT) by wheat and soybean cell suspension cultures yielded similar metabolite classes as reported in whole plants, although proportions of metabolite classes differed (Sandermann et al. 1984). Additionally, Babczinski (1999) observed “very rare” plant metabolites, including a 2'-*O*-sulfated glycoside and a 2'-*O*-linked glucuronic acid containing disaccharide, in red

beet cell suspension cultures; as cell suspension studies were not conducted in parallel with whole plant studies, verification of the “very rare” plant metabolites in whole plant systems was not possible.

Plants from the family of Lemnaceae (commonly known as duckweed) possess great potential for comparisons of pollutant toxicity and metabolism in whole plants and in plant tissues culture. Lemnaceae plants are morphologically simple and grow rapidly via asexual reproduction. Additionally, Lemnaceae species, especially *Lemna gibba* and *Lemna minor*, are commonly used to assess toxicity of organic pollutants, including emerging pollutants like fluorinated organic pollutants, pharmaceuticals, and personal care products (EPA 1996; ASTM 1997; Pro et al. 2003; Brain et al. 2004a). Lemnaceae species actively uptake many organic pollutants, including chlorinated and fluorinated phenols (Reinhold and Saunders 2006b; Tront et al. 2007) and pharmaceuticals and personal care products (Chapter 6). With a cosmopolitan distribution, multiple Lemnaceae species frequently grow in similar aquatic habitats. Consequently, Lemnaceae species are ideal model plants for examining relationships between toxicities and metabolism of organic pollutants to whole plants and plant tissue cultures. However, reports of successful culture of Lemnaceae cell suspensions were not found after extensive literature review, although many studies have examined induction and growth of Lemnaceae callus (Chang and Chiu 1976; Frick 1991; Moon and Stomp 1997; Li et al. 2004).

Previous studies on phytotoxicity and phytometabolism of pollutants in plant cell suspension cultures suggest a role for Lemnaceae callus cultures in researching toxicity and environmental fate of pollutants. However, callus cultures possess characteristics that may affect their interactions with organic pollutants. Variability with respect to structural, metabolic, and genetic features is a crucial characteristic of plant callus that results from an absence of control systems that operate in the whole plant (Warren 1992). Initiation and culture of Lemnaceae callus frequently results in multiple types of callus,

including yellow, irregularly shaped, friable, and fast growing callus and green, compact, and slow growing callus (Chang and Chiu 1976; Moon and Stomp 1997). Consequently, variability of responses to organic pollutants may be inherent to callus culture assessments. Additionally, contact of callus with solidified medium is limited and mass transfer limitations, between callus and medium and within the medium itself, may affect callus responses to pollutants. Finally, callus culture growth requires complex medium containing vitamins, phyto regulators, and carbon sources that may affect plant interactions with pollutants or themselves interact with pollutants. Therefore, comparison of callus cultures to plants in model species is crucial for further development of callus assessments of phytotoxicity and phytometabolism of organic pollutants.

Phytotoxicity depends on internal concentration of organic pollutant – consequently, a discussion of processes contributing to or subtracting from internal concentration of pollutant within callus or whole plant cultures is warranted. Internal pollutant concentration in Lemnaceae plants is determined by rates of partitioning into plant tissues and rates of metabolism of organic pollutants. Rate of partitioning into Lemnaceae plants depends on external aqueous concentration of pollutant, which in turn depends on abiotic and biotic processes that affect concentration, including diffusion through aqueous medium, volatilization, microbial degradation, and plant uptake. Likewise, internal pollutant concentration in Lemnaceae callus depends on rates of partitioning into callus and rates of metabolism of organic pollutants by Lemnaceae callus. However, partitioning of pollutants into callus may be limited, as the surface area to volume ratio in contact with medium is substantially less. Exposure of callus to pollutants may also depend on pollutant volatility; the more spherical shape of callus, as compared to flat oval shape of Lemnaceae fronds, may allow for significant diffusion from the gaseous phase into callus or moisture on callus surfaces that are not in direct contact with solidified medium. Volatilization may also contribute to pollutant loss from callus cultures, subsequently decreasing exposure of callus to organic pollutants.

Metabolism of organic pollutants, and subsequent decrease in internal concentration and inhibitory effects of organic pollutants, may also differ in Lemnaceae plants and callus cultures.

Previous studies using non-organ plant tissues cultures to determine phytotoxicity and phytometabolism of pollutants generally utilized well-established (e.g., > 10 years) cell suspension cultures (Sandermann et al. 1984). Extensive literature review did not yield reports of applications for plant callus cultures, particularly cultures of aquatic plant species, for phytotoxicity and phytometabolism studies. Therefore, the primary aim of research presented herein was to compare toxicity and metabolism of fluorinated organic pollutants in Lemnaceae and Lemnaceae callus. In contrast to previous studies, callus was cultured for less than six months prior to toxicity and metabolism studies. Consequent objectives were to (1) develop descriptive parameters for callus growth and inhibition from pollutants, (2) assess qualitative and quantitative applicability of callus toxicity assessments to whole plant toxicities, and (3) compare phytometabolism of 3-trifluoromethylphenol in callus and whole plant cultures.

Material and Methods

Collection and callus initiation of Lemnaceae

Lemnaceae communities were collected from a tertiary treatment wetland that reclaims wastewater effluent for water reuse in Clayton County, GA. Species were separated and identified using 10 to 40X light and phase contrast microscopy with an internet dichotomous key (Armstrong 2001). Species identification indicated that *Lemna minor* and *Landoltia punctata* were the dominant Lemnaceae species in the treatment wetland. Prior to fluorinated organic pollutant exposure and callus initiation, Lemnaceae communities were cleaned, via rinsing and manual removal of debris and insects, and were maintained in Standard Methods medium (APHA et al. 1998b) under a 16-8 h light:

dark photoperiod.

Callus cultures of *L. minor* and *L. punctata* were initiated with previously described procedures (Moon and Stomp 1997; Li et al. 2004). In brief, *L. minor* callus was initiated and maintained from disinfected fronds on Mushirage and Skoog medium containing 3% sucrose, 45 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.92 μM N⁶-(3-methylbut-2-enyl)adenine (2iP). *L. punctata* callus was initiated from disinfected fronds on medium containing ½ Mushirage and Skoog basal salts and Gamborg (B5) vitamins, with 15.8 μM 2,4-D, 9.84 μM 2-iP, 67.9 μM dicamba, and 1% sorbitol. *L. punctata* callus was maintained on Woody Plant medium containing 4.52 μM 2,4-D, 26.9 μM 1-naphthalene acetic acid (NAA), 2.27 μM thiadizuron (TDZ), and 2% sorbitol. Both species of callus were maintained under partial (*L. minor*) or continuous light (*L. punctata*). Callus was cultured for approximately six months prior to toxicity and metabolism assessments to allow for selection of stable callus cultures and minimization of habituation or somaclonal variation that could result from long-term culture. *L. minor* cultures consisted primarily of friable white and yellow callus, although more compact, medium green callus was frequently observed. Two *L. punctata* callus types, representative of *L. punctata* callus cultures 804/808 and 815, were observed and utilized in toxicity assessments. *L. punctata* 804/808 callus was light green and bulbous, while *L. punctata* 815 callus was dark green and compact. Representative photos of callus cultures are provided in Figure 8.1.

Fluorinated organic pollutants

Fluorinated organic pollutants, primarily fluorinated and trifluoromethylated phenols, were initially screened with single oxygen production rate assessments to allow for selection of experimental pollutants with a broad range of toxicities. Selected experimental pollutants were two trifluoromethylated phenols (i.e., 2-trifluoromethylphenol [2-TFMeP] and 3-trifluoromethylphenol [3-TFMeP]), two

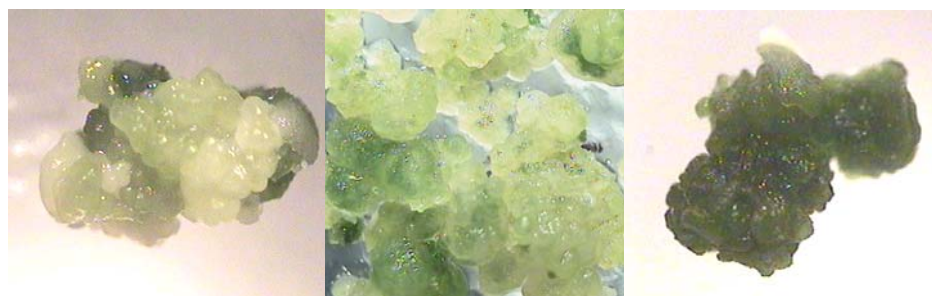


Figure 8.1. Examples of *L. minor*, *L. punctata* 804/8, and *L. punctata* 815 callus cultures (left to right, respectively).

fluorophenols (i.e., 2,4-difluorophenol [2,4-DFP] and 2,3,5-trifluorophenol [2,3,5-TFP]), and fluoxetine (FLX). Concentration ranges were designed so that multiple concentrations for each fluorinated organic pollutant were within 20% to 80% inhibition of Lemnaceae populations (as indicated by preliminary whole-plant toxicity assessments). Consequently, toxicity assessments utilized concentrations ranging from 100 – 1500 μM 2-TFMeP, 100 – 1500 μM 2,4-DFP, 10 – 2000 μM 3-TFMeP (trial 1), 100 – 500 μM 3-TFMeP (trial 2), 50 – 250 μM 2,3,5-TFP, and 2.3 – 100 μM FLX, with a minimum of five concentrations increments per experimental pollutant. Additionally, no-pollutant controls were utilized for each combination of pollutant and callus species/line.

Oxygen production rate (OP) assessment

Lemnaceae, specifically *L. minor* and *L. punctata*, were exposed to experimental concentrations of fluorinated organic pollutants for 2 d under continuous light. Exposure reactors consisted of 250-mL Erlenmeyer flasks with 100 mL Standard Methods (SM) (APHA et al. 1998b) medium buffered at pH 5.8 with 10 mM phosphate buffer, experimental concentration of fluorinated organic pollutant, and 1.5 g of either *L. minor* or *L. punctata*. pH measurements indicated negligible (<0.2) change in pH during pollutant exposure. Surface coverage of Lemnaceae, at 1.5 g per 100 mL of medium, was greater than 100%, thus reflecting growth conditions of Lemnaceae observed at

constructed treatment wetlands. Full surface coverage of duckweed also limits volatilization of pollutant from reactors; additionally, small plates were placed over mouths of reactors to minimize evaporation and volatilization. Desired quantities of fluorinated organic pollutants were added from acetonitrile or ethanol stocks after determining that addition of acetonitrile or ethanol to exposure reactors did not affect whole-plant activity (measured as described subsequently). Exposure reactors, and subsequent measures of whole-plant activity and inhibition, were completed in triplicate.

Whole-plant Lemnaceae activity and inhibition was assessed using oxygen production rate (OP) analysis as described previously (Reinhold and Saunders 2006a) at conclusion of exposure of Lemnaceae to fluorinated organic pollutants. Briefly, two sealed reactors were incubated at 22 °C, with one exposed to the light and one maintained in the dark for each replicate analysis. Reactors consisted of 160-mL serum bottles with 60-mL modified Standard Methods (APHA et al. 1998b) medium and 0.5 g of Lemnaceae. At the end of incubation, change in total headspace volume at atmospheric pressure was measured with manometers and headspace oxygen was analyzed by gas chromatography with thermal conductivity detector. The μmol of oxygen produced by Lemnaceae was calculated by subtracting oxygen in the dark reactor ($\mu\text{mol O}_{2,\text{dark}}$) from the oxygen in the light reactor ($\mu\text{mol O}_{2,\text{light}}$), where the dark reactor accounted for oxygen consumed by respiratory processes. Consequently, oxygen production assessments measured total oxygen produced by photosynthesis. Oxygen production rate (OP) was calculated by normalizing oxygen produced with incubation time (Δt) and initial fresh mass of Lemnaceae, yielding units of $\mu\text{mol O}_2/\text{h-g Lemnaceae}$:

$$OP = \frac{\mu\text{mol O}_{2,\text{light}} - \mu\text{mol O}_{2,\text{dark}}}{M \cdot \Delta t} \quad \text{Equation 8.1}$$

Toxicity of fluorinated organic pollutants to Lemnaceae was characterized by the concentration at which 50% of OP was inhibited (EC50) and the lowest concentration at which inhibition was observed (LOEC). EC50 values were determined with 3-parameter

logistic sigmoid relationships using Sigma Stat 3.1 (Systat Software Inc. 2004):

$$OP = \frac{a}{1 + \left(\frac{C}{EC50}\right)^b} \quad \text{Equation 8.2}$$

where a and b are fitting parameters. LOEC values were determined with one-way analysis of variance (ANOVA; $p < 0.001$ indicating significant difference in OP values). To facilitate comparisons of inhibition of Lemnaceae independent of control OP, OPs for experimental concentrations of fluorinated organic pollutant (C , x μM) were normalized to OPs for control Lemnaceae:

$$\beta = \frac{OP_{x \mu\text{M}}}{OP_{0 \mu\text{M}}} \quad \text{Equation 8.3}$$

Control reactors for β calculations were conducted in parallel with toxicity assessments and consequently accounted for variability of Lemnaceae activity based on species, age, health, and any other conditions not related to fluorinated organic pollutant exposure.

Callus toxicity assessment

Callus toxicity assessments utilized respective solidified callus growth medium for *L. minor* and *L. punctata* callus. Medium was autoclaved prior to addition of fluorinated organic pollutant to eliminate potential thermal degradation of fluorinated organic pollutants. Stock fluorinated organic pollutant, in acetonitrile or ethanol, was added to achieve desired experimental concentrations. Preliminary studies indicated that acetonitrile or ethanol addition, in quantities required to achieve experimental fluorinated organic pollutant concentrations, did not adversely or beneficially affect callus growth. Assessments of callus toxicity utilized two 24-well plates (2 mL medium per well) for each treatment (i.e., concentration x fluorinated organic pollutant x callus culture). Additionally, control callus growth was measured in two 24-well plates with no fluorinated organic pollutants for each combination of fluorinated organic pollutant and callus culture.

Prior to placement on callus toxicity assessment medium, callus was carefully scraped through a no. 11 stainless steel sieve into a Petri dish containing autoclaved E-pure water. Callus preparation utilized no. 11 sieve because 2-mm opening produced moderately uniformly sized callus that was of sufficient size for continued growth. Callus achieved via separation with smaller sieves (i.e., 1.0 and 1.4 mm openings) in preliminary studies were not sufficiently large enough to prevent senescence. After separation of callus, calluses were selected for toxicity assessments based on observations at 20X light microscopy. Criteria for callus selection were absence of differentiated/organized tissues and healthy color of white, yellow, or green. Selected calluses were plated on toxicity assessment medium sequentially to minimize selection-effects on assessments (e.g., callus placed on all A1 wells for all concentrations prior to any callus placement A2 wells, for all 24 wells). Additionally, order of plates was rotated after placement of four to six callus (depending on number of concentration increments). Finally, callus preparation and placement for each treatment were completed in two separate batches, with each batch representing one 24-well plate. Callus toxicity assessments utilized 4-wk exposures, as 4-wk was maximum incubation period before transfer of callus was required.

Callus was individually photographed at 10X to 30X at the beginning and conclusion of toxicity assessments utilizing Motic digital stereo zoom microscope. Callus images were analyzed with Motic Images Plus 2.0 ML for initial and final surface areas of each callus. Microscope was calibrated utilizing calibration circles elevated to approximate level of callus toxicity assessment medium. Automatic integration function had difficulty distinguishing between callus and solidified medium, especially when callus was lightly colored or senescent. Consequently, callus outlines were manually traced prior to software integration of surface area (A) in mm^2 .

Multiple growth descriptive parameters were analyzed for use in callus toxicity assessments, including linear growth rates, logarithmic growth rates, final surface area

(A_f), and log of final surface area. Parameters were evaluated based on distribution and independence on initial callus surface area. Distributions of parameters were deemed normal based on Kolmogorov-Smirnov test (i.e., $p > 0.200$ and K-S distribution factor < 0.05). Independence of parameters on initial callus surface areas was determined utilizing t-tests (i.e., $p < 0.001$).

Toxicity of fluorinated organic pollutants to Lemnaceae was characterized by the concentration at which 50% of callus growth was inhibited (EC50) and the lowest concentration at which inhibition was observed (LOEC). EC50 values were determined by fitting log of final callus surface areas ($A_{F,x \mu M}$) and concentration (C) of fluorinated organic pollutants with four-parameter logistic sigmoid relationships using SigmaStat 3.11 (Systat Software Inc. 2004):

$$\log A_{F,x \mu M} = \log A_d + \frac{a}{1 + \left(\frac{C}{EC50}\right)^b} \quad \text{Equation 8.4}$$

where a and b are fitting parameters and A_d is final surface area of senescent callus. Log A_d was experimentally calculated from mean values of log $A_{F,x \mu M}$ in callus culture-specific treatments where callus senescence and net reduction in surface area was observed. Net reduction in surface area was assumed to result from water loss from senescent callus. Log A_d values were 0.36 ± 0.03 , 0.55 ± 0.02 , and 0.56 ± 0.01 (mean \pm standard error) for *L. minor*, *L. punctata* 804/808, and *L. punctata* 815. Statistical validity was concluded for fits with $p < 0.01$ and $r^2 > 0.80$. LOEC values were determined with one-way analysis of variance (ANOVA; $p < 0.001$ indicating significant difference in callus growth).

To allow additional comparison between assessments, species, callus cultures, and pollutants, percentage of callus growth was normalized to controls and the average loss of senescent callus surface area was incorporated into normalization. The resulting normalized callus growth factor, γ , was calculated with the following equation:

$$\gamma = \frac{\log A_{F,x\mu M} - \log A_d}{\log A_{F,0\mu M} - \log A_d} \quad \text{Equation 8.4}$$

where $A_{F,x\mu M}$ is final callus surface area at x μM fluorinated organic pollutant and $\log A_d$ and $\log A_{F,0\mu M}$ were empirical constants calculated from experimental data. $\log A_{F,0\mu M}$ values were mean values of the log of final callus surface area at 0 μM of fluorinated organic pollutant and were callus culture and fluorinated organic pollutant specific.

Identification of plant metabolites of 3-TFMeP

To compare metabolic pathways of 3-TFMeP in Lemnaceae plants and callus cultures, Lemnaceae cultures were exposed to 50 μM 3-TFMeP. *L. minor* and *L. punctata* populations (25 g) were exposed to 50 μM 3-TFMeP in 1 L of SM medium in 2-L Erlenmeyer flasks for 5 days. Aqueous concentrations at conclusion of 3-TFMeP exposure were 10.0 μM and 30.3 μM in *L. minor* and *L. punctata* reactors, respectively. *L. minor* and *L. punctata* 804/808 callus cultures were exposed to 50 μM 3-TFMeP for 8-wk. Cultures were transferred to fresh medium with 50 μM 3-TFMeP after 4 wks.

Extraction procedures for identification of metabolites utilized procedures developed for 2,4-dichlorophenol and 2,4,5-trichlorophenol by *L. minor* (Day and Saunders 2004). Plants and callus cultures were frozen at -80°C at conclusion of 3-TFMeP exposure. Frozen plant and callus tissues were ground to fine powder in pre-cooled mortars. Ground tissues were extracted 3-times with 5-mL of 80% acetonitrile per gram of tissue, utilizing centrifugation for separation of tissue solids from extracts. Crude extracts were purified with solid phase extraction (SPE) prior to mass spectral analysis. Briefly, 100 mL of crude extract was eluted on 10g x 60 mL Supelco LC-18 SPE tubes to reduce concentration of chlorophyll and other chemicals that could interfere with metabolite analysis. SPE tubes were preconditioned with 50 mL acetonitrile followed by 50 mL 10% acetonitrile in water by volume. After loading of crude extracts, SPE tubes were flushed by gravity with 50 mL acetonitrile. Extract load and flush were

combined for analysis.

Purified extracts were analyzed using liquid chromatography and mass spectrometry (LC-MS) on Agilent 1100 series LC-DAD-MSD. Metabolites and plant compounds were separated using a gradient starting with 20% aqueous mobile phase (0.1% acetic acid) and ending with 100% organic mobile phase (acetonitrile) on a reverse phase STABLE BOND-C18 analytical column (2.1x150 mm, 5 μ m, Agilent). Electrospray ionization, in the negative mode, was used for identification of metabolites and parent 3-TFMeP. To screen for conjugated metabolites of 3-TFMeP, chromatograms were screened for a m/z signal of 161 by extracting 161 from the total ion chromatogram.

Results and Discussion

Characterization of control callus growth

Initial callus surface areas were statistically distinct for *L. minor*, *L. punctata* 804/808, and *L. punctata* 815 (i.e., $p < 0.05$ for all pair-wise comparisons). *L. minor* callus cultures possessed the smallest initial surface areas of $3.21 \pm 0.06 \text{ mm}^2$. *L. punctata* callus cultures were significantly larger, with initial callus surface areas of $3.97 \pm 0.08 \text{ mm}^2$ for *L. punctata* 804/808 and $4.31 \pm 0.07 \text{ mm}^2$ for *L. punctata* 815. Friability was likely the main contributor to decreased initial callus surface areas for *L. minor*, as *L. minor* callus was substantially more friable and more easily separated into smaller pieces via scraping through a 2-mm screen. Additionally, distribution characteristics of initial callus surface areas depended on callus culture (Figure 8.2). Initial surface areas of *L. minor* callus cultures were normally distributed as indicated by Kolmogorov-Smirnov (K-S) distribution factor of 0.048 (i.e., $p > 0.20$). However, initial surface areas of *L. punctata* 804/808 and *L. punctata* 815 callus cultures were not normally distributed (i.e., K-S factors of 0.092 and 0.079, respectively; $p < 0.01$). Non-normal distributions for *L. punctata* 804/808 and *L. punctata* 815 resulted from relatively high skewness (i.e., $g_1 =$

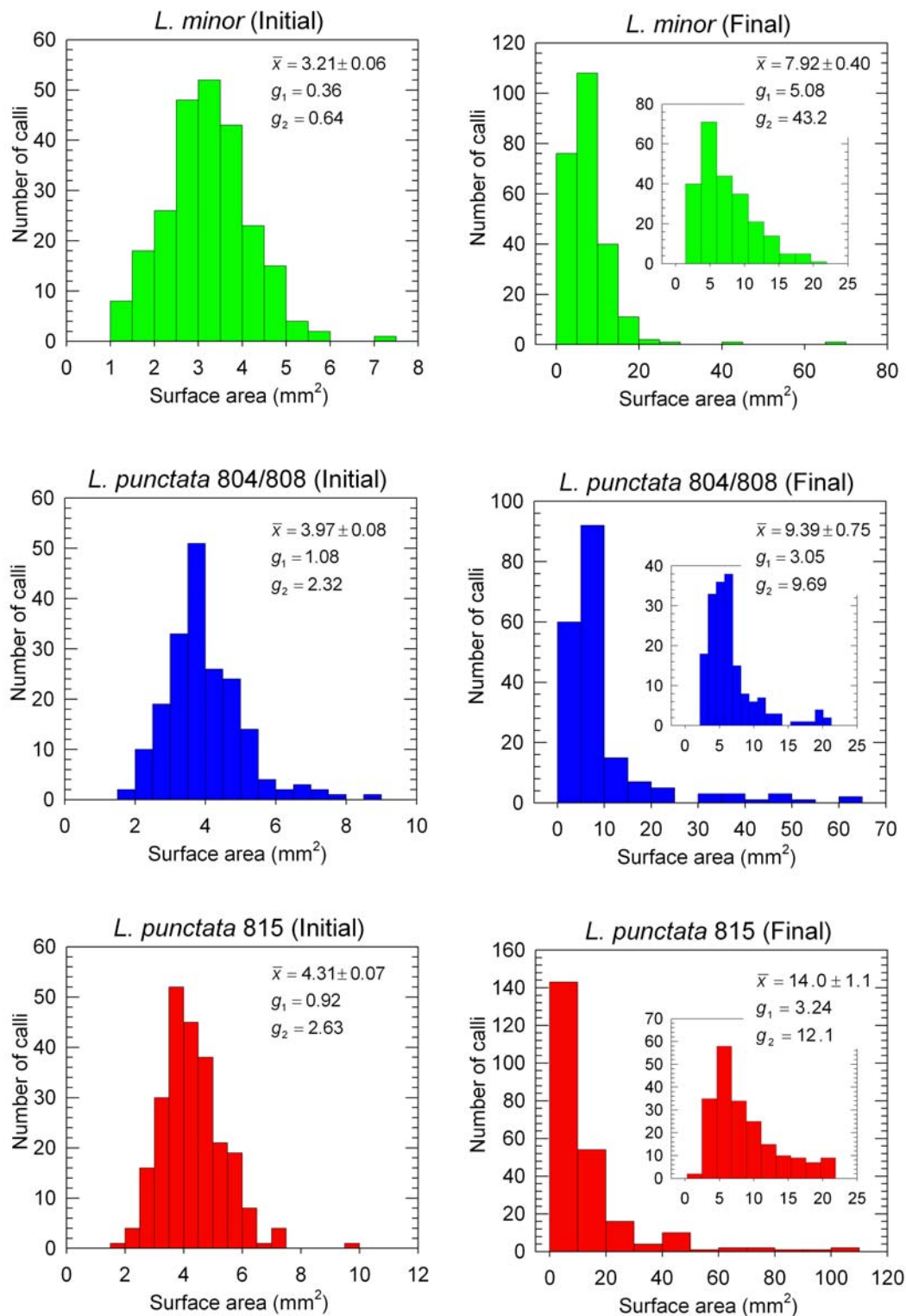


Figure 8.2. Distributions of initial and final surface areas for *L. minor*, *L. punctata* 804/808, and *L. punctata* 815 in experimental controls (no exposure to fluorinated organic pollutant). Means (\bar{x}) are provided with standard errors, skewness factors (g_1) and Kurtosis factors (g_2).

1.08 and 0.92, respectively) and kurtosis (i.e., $g_2 = 2.32$ and 2.63 , respectively). In contrast, skewness and kurtosis of initial *L. minor* callus surface areas were significantly less (i.e., $g_1 = 0.36$ and $g_2 = 0.64$). Combined higher, positive kurtosis and positive skewness in distribution of initial callus surface areas indicates that majority of callus cultures possessed initial surface areas very similar to median initial surface area, but that remaining calluses were characterized by substantially greater initial surface areas. Positively kurtotic and skewed distributions suggest that manual scraping of callus through 2-mm screen produced relatively narrow size distribution of callus (e.g., ~ 3 to ~ 5 mm²), with notable exceptions of a few large callus pieces (e.g., > 6 mm²). It is also highly probable that subjectivity of selection increased the positive skew of initial callus surface area distributions, as larger callus pieces were likely considered “healthier” or “better” by researchers selecting callus.

The subjective assumption that larger calluses would exhibit hardier or more rapid growth was invalidated by a lack of correlations between initial and final callus surface areas in controls. For 9 of 14 data sets, control callus growth was strongly independent of initial callus surface area (i.e., $r^2 < 0.10$). Remaining data sets demonstrated slightly weaker independence on final surface area on initial surface area (i.e., 3 data sets with $0.10 < r^2 < 0.30$ and 2 data sets with $0.30 < r^2 < 0.50$). Final callus surface area was more likely to depend on initial callus surface area (i.e., $0.30 < r^2 < 0.50$) when callus growth was minimal, as indicated by less than 50% increase in callus size after 4 wk.

Skewness and kurtosis of callus surface area distributions increased with growth of callus, as shown in Figure 8.2. Consequently, observed increases of 250%, 240%, and 320% for mean *L. minor*, *L. punctata* 804/8, and *L. punctata* 815 callus surface areas (respectively) were greater than observed increases of 200%, 150%, and 200% for median callus surface areas (respectively). Increase in skewness and kurtosis of size distributions with callus growth strongly indicates that callus growth was inherently not normally distributed and that mathematically transformations of final and initial surface

areas were required prior to applying standard statistical techniques, such as one-way ANOVA or student *t*-tests. While rank based statistical techniques could be applied to raw data (e.g., ANOVA on ranks or rank sum tests), closer examination of data revealed that use of median values to represent growth frequently yielded regressions and analysis that were not graphically representative of callus toxicity assessment data or visual observations of callus growth and senescence.

Independence of growth descriptive parameters on initial surface area and normal distribution of parameters for each treatment were crucial criteria for selection of growth descriptive parameters for callus toxicity assessments. The log of final callus surface area ($\log A_f$) was best descriptor of callus growth in toxicity assessments when compared with both linear and logarithmic growth rates. Although previous studies have indicated that both linear and logarithmic growth rates were statistically valid descriptions of *L. minor* and *L. punctata* callus growth (Handell 2007), measurements of linear and logarithmic growth rates in callus toxicity assessments were not normally distributed. Linear growth rates, represented by percentage increase in callus surface area (%SA), were independent of initial callus surface areas, while logarithmic growth rates depended on initial callus surface area (i.e., $r^2 > 0.50$). However, $\log A_f$ values were normally distributed and independent of initial surface areas (and log of initial surface areas).

Intercomparisons of callus toxicity responses

Toxicity of experimental fluorinated organic pollutants to Lemnaceae callus cultures ranged from inhibition of *L. punctata* 815 at 50 μ M fluoxetine to no inhibition of *L. minor* callus at <1500 μ M 2,4-DFP. Table 8.1 summarizes the fitted EC50 and LOEC values for 2-TFMeP, 3-TFMeP, 2,4-DFP, 2,3,5-TFP, and fluoxetine.

Table 8.1. Representative parameters for description of toxicity responses of *L. minor* and *L. punctata* to fluorinated organic pollutants, as determined by callus and oxygen production (OP) rate assessments. Concentrations (μM) at which 50% inhibition of callus growth or OP was observed (EC50s) and minimum concentrations at which inhibition of callus growth or OP were observed (LOECs) are provided with r^2 and/or p values.

Pollutant		Assessment with callus				Assessment with OP				
Species CL ^a	EC50 (μM)	r^2	p -value	LOEC (μM)	p -value	EC50 (μM)	r^2	p -value	LOEC (μM)	p -value
<u>2-Trifluoromethylphenol</u>										
<i>L. minor</i>	517±374	0.989	0.0158	100	<0.05	1260±46	0.946	<0.001	1000	<0.05
<i>L. punctata</i> 804	880±285	0.994	0.0088	500	<0.05					
<i>L. punctata</i> 815	639±188	0.976	0.0062	500	<0.05	1799±302	0.876	<0.001	1500	<0.05
<u>3-Trifluoromethylphenol</u>										
<i>L. minor</i>	143±39	0.940	<0.0001	100	<0.05					
<i>L. minor</i> ^b	319±89	0.966	<0.0001	100	<0.05	>500	--	--	>500	0.627
<i>L. punctata</i> 808	390±43	0.950	<0.0001	500	<0.05					
<i>L. punctata</i> 815	482±182	0.948	<0.0001	500	<0.05					
<i>L. punctata</i> 815 ^b	154±20	0.952	<0.0001	200	<0.05	>500	--	--	400	<0.05

Table 8.1 Continued

Pollutant		Assessment with callus				Assessment with OP				
Species CL ^a	EC50 (μM)	r^2	<i>p</i> -value	LOEC (μM)	<i>p</i> -value	EC50 (μM)	r^2	<i>p</i> -value	LOEC (μM)	<i>p</i> -value
<u>2,4-Difluorophenol</u>										
<i>L. minor</i>	>2000	NC	NC	1500	<0.05	>2000	--	--	2000	<0.05
<i>L. punctata</i> 804	792±335	0.956	<0.0001	250	<0.05					
<i>L. punctata</i> 815	541±51	0.971	<0.0001	500	<0.05	1739±115	0.888	<0.001	1000	<0.05
<u>2,3,5-Trifluorophenol</u>										
<i>L. minor</i>	77.1±4.2	0.952	0.0010	100	<0.05	212±19	0.873	<0.001	100	<0.05
<i>L. punctata</i> 815	72.2±5.2	0.977	<0.0001	100	<0.05	181±23	0.879	<0.001	100	<0.05
<u>Fluoxetine</u>										
<i>L. minor</i>	>100	NC	NC	>100	0.38	34.7±7.5		<0.001	15	<0.05
<i>L. punctata</i> 815	56.4±27.3	0.936	<0.0001	50	>0.05	89.7±5.4		<0.001	50	0.07

^a Results from two callus cultures of *L. punctata* (804/808 and 815) are provided. *L. punctata* callus cultures were either compact and dark green (804 and 808) or non-compact and pale green (815).

^b Assessments of increase in callus surface areas was repeated for 3-TFMeP with narrower range of concentrations (i.e., 10 – 2000 μM vs. 100 – 400 μM 3-TFMeP).

One notable observation of EC50 values presented in Table 8.1 is significant differences in EC50 values derived for 3-TFMeP in first and second trials (i.e., 142 ± 39 μM vs. 319 ± 89 μM for *L. minor* and 482 ± 182 μM vs. 154 ± 20 for *L. punctata* 815, respectively). Initial callus assessments with 3-TFMeP and 2,3,5-TFMeP yielded rapid transition from no observed effects to complete toxicity, as indicated by callus senescence. Examples of these trends are provided for reference in Table 8.1 for initial trials of *L. punctata* 808, *L. punctata* 815, and *L. minor* callus exposed to 3-TFMeP, where LOEC values were greater than EC50 values. These results necessitated repetition of 3-TFMeP and 2,3,5-TFP callus toxicity assessments with lower concentrations of 100 to 400 μM 3-TFMeP and 50 to 200 μM 2,3,5-TFP. While varying increments of experimental concentrations in first and second trials contributed to variation in EC50 values, a notable difference in response of *L. minor* to 3-TFMeP in first in second trials was observed. Inhibition of *L. minor* callus by 100 μM 3-TFMeP, as quantified by γ , was significantly greater in the second trial than in the first trial (i.e., 0.291 ± 0.172 and 0.822 ± 0.033 , respectively; $p=0.030$). However, *L. punctata* 815 callus responded similarly to 100 μM 3-TFMeP in both trials, with γ values of 0.863 ± 0.050 and 0.717 ± 0.106 , respectively (i.e., $p=0.215$). Increased reproducibility of results for *L. punctata* 815 callus assessments, as compared to *L. minor* assessments, may have resulted from differing levels of heterogeneity associated with *L. punctata* 815 and *L. minor* callus. *L. punctata* 815 callus was largely homogeneous, consisting of compact, dark green callus that remained predominantly dedifferentiated and unchanged in morphology during the four-week callus assessment. In contrast, *L. minor* cultures consisted of heterogeneous composites of friable light yellow or white callus and more compact green callus. While callus did not re-differentiate into organized structures during 4-week toxicity assessment, callus growth frequently consisted of both types, yielding heterogeneous cultures even when starting material consisted of one type of callus. Therefore, difference in response of *L. minor* callus to 100 μM 3-TFMeP may be

attributed to heterogeneous nature of *L. minor* callus. However, it is important to note that the difference was slight (i.e., $p=0.030$ vs. $p>0.050$) and the same LOEC values were obtained by both assessments (i.e., 100 μM). Therefore, while the slight difference in *L. minor* response to 100 μM 3-TFMeP in separate exposures highlights the importance of callus homogeneity in toxicity assessments, the difference is not substantial enough to negate use of *L. minor* callus for toxicity assessments. Furthermore, analysis of γ values for first and second exposures of callus to 100 μM 3-TFMeP strongly indicates that experimental design, specifically improved selection of concentration values for the second 3-TFMeP trial, is the major contributor to observed differences in EC50 values for first and second 3-TFMeP toxicity assessments.

As indicated by EC50 values, both types of *L. punctata* callus responded similarly to fluorinated organic pollutant exposure. Values for EC50 were statistically similar for *L. punctata* 804/808 and *L. punctata* 815 callus assessments (e.g., 880 ± 285 μM vs. 639 ± 188 μM 2-TFMeP, 390 ± 43 μM vs. 482 ± 182 μM 3-TFMeP, and 792 ± 335 μM vs. 541 ± 51 μM 2,4-DFP). Additionally, similar LOEC values of 500 μM 2-TFMeP and 500 μM 3-TFMeP were determined for *L. punctata* 808 and *L. punctata* 815 callus. LOEC values for 2,4-DFP were less for *L. punctata* 804 than for *L. punctata* 815, though the difference was only one concentration increment (i.e., 250 μM vs. 500 μM 2,4-DFP). Therefore, toxicity parameters derived from multiple callus cultures from the same species were deemed reasonably similar.

When γ values were utilized to examine the relationship between *L. punctata* 804/808 and *L. punctata* 815 responses to individual concentrations of fluorinated organic pollutants, results strongly indicated that responses were not specific to callus culture within one species. As shown in Figure 8.3, *L. punctata* 804/8 and *L. punctata* 815 exhibited similar responses to multiple concentrations of fluorinated organic pollutants, as measured by γ -values.

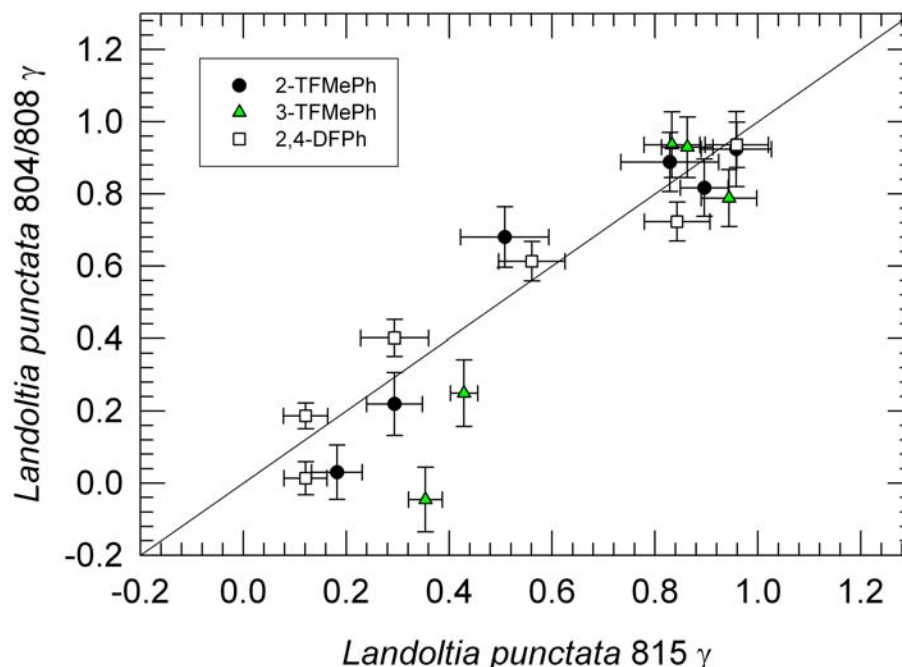


Figure 8.3. Comparison of normalized responses of *L. punctata* 804/808 callus to normalized responses of *L. punctata* 815, as represented by γ , for 2-trifluoromethylphenol (2-TFMePh), 3-trifluoromethylphenol (3-TFMePh), and 2,4-difluorophenol (2,4-DFP). Solid line represents $\gamma_{LP\ 804/8} = \gamma_{LP815}$. Points represent mean values and error bars represent standard error of 48 replicates.

Values for γ were statistically similar for both *L. punctata* callus cultures (i.e., $p=0.897$).

Linear regression of *L. punctata* 804/8 γ and *L. punctata* 815 γ values further indicated that responses of *L. punctata* callus cultures to fluorinated organic pollutants were similar, as resulting linear regression of $\gamma_{LP\ 804/8} = (-0.08 \pm 0.08) + (1.07 \pm 0.11) \gamma_{LP815}$ ($r^2 = 0.89$) was statistically similar to $\gamma_{LP\ 804/8} = \gamma_{LP815}$. Similarity between γ -values for both *L. punctata* callus types demonstrated reproducibility of callus toxicity results for callus cultures from a given species of callus.

L. minor callus was generally less or equally susceptible to inhibition of callus growth by fluorinated organic pollutants than was *L. punctata* 815 callus. EC50 values were statistically similar for two fluorinated organic pollutants, as indicated by EC50 values of $517 \pm 374\ \mu\text{M}$ vs. $638 \pm 188\ \mu\text{M}$ 2-TFMePh and $77.1 \pm 4.2\ \mu\text{M}$ vs. $72.2 \pm 5.2\ \mu\text{M}$

2,3,5-TFP for *L. minor* and *L. punctata* 815, respectively. For three fluorinated organic pollutants, EC50 values for *L. minor* callus of $319 \pm 89 \mu\text{M}$ 3-TFMeP, $>2000 \mu\text{M}$ 2,4-DFP, and $>100 \mu\text{M}$ FLX were significantly greater than corresponding EC50 values for *L. punctata* 815 callus of $154 \pm 20 \mu\text{M}$ 3-TFMeP, $541 \pm 51 \mu\text{M}$ 2,4-DFP, and $>100 \mu\text{M}$ FLX. Interestingly, decreased susceptibility to fluorinated organic pollutants exhibited by *L. minor* callus was not apparent in LOEC values for 2-TFMeP and 3-TFMeP, which were lower than corresponding LOEC values for *L. punctata*.

Additionally, γ values for *L. minor* and *L. punctata* 815 callus were neither similar nor correlated (Figure 8.4). Correlation between *L. minor* γ -values and *L. punctata* γ -values was low ($r^2 = 0.48$). Specificity of γ values to *L. minor* and *L. punctata* callus cultures was most apparent in callus culture toxicity assessments of fluoxetine.

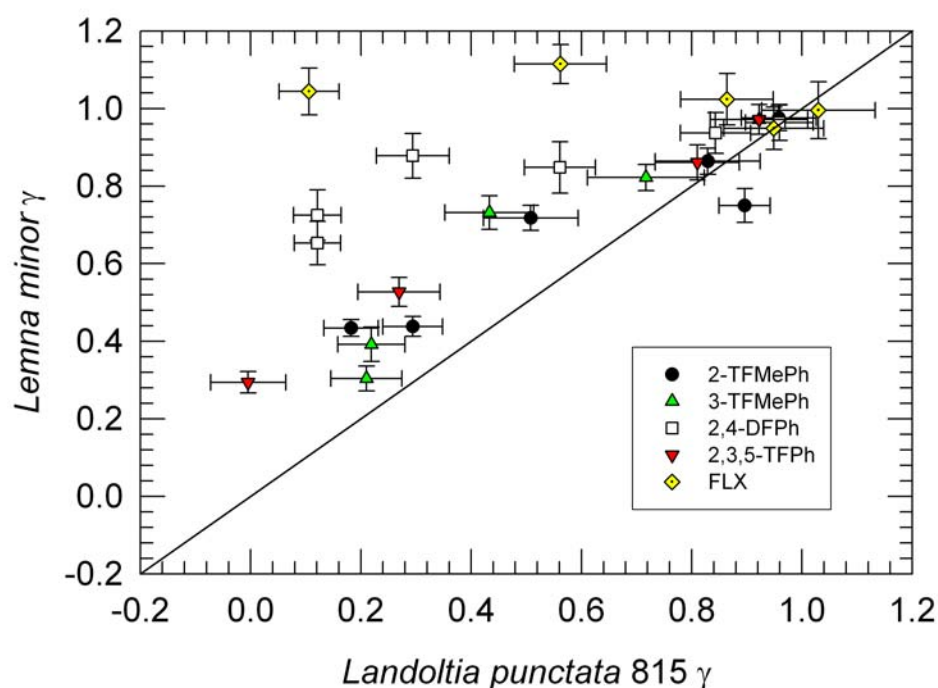


Figure 8.4. Comparison of normalized responses of *L. minor* callus to normalized responses of *L. punctata* 815, as represented by γ , for fluorinated organic pollutants. Solid line represents $\gamma_{\text{LM}} = \gamma_{\text{LP 815}}$. Points represent mean values and error bars represent standard error of 48 replicates.

L. minor was not inhibited by fluoxetine at any experimental conditions (i.e., $0.95 \leq \gamma_{LM} \leq 1.11$), while *L. punctata* was inhibited at $>50 \mu\text{M}$ fluoxetine (i.e., $0.11 \leq \gamma_{LP815} \leq 1.03$). Increases in γ values were observed for both *L. minor* and *L. punctata* 815 callus cultures at $5 \mu\text{M}$ fluoxetine, indicating the possibility of some stimulatory effects of low concentrations of fluoxetine on callus growth. Dependence of γ values on callus species suggests that callus cultures maintained species-specificity; however, it is important to note that composition of callus medium should not be ruled out as a contributing factor to specificity of callus culture toxicity assessments. Callus growth medium for *L. minor* and *L. punctata* differed in regards to pH (5.6 vs. 5.8, respectively), carbon source (sucrose vs. sorbitol, respectively), basal nutrient medium (MS vs. WP, respectively), and phytohormones (2,4-D and 2-iP vs. 2,4-D, NAA, and TDZ, respectively). Difference in pH was considered negligible, but other medium components could potentially be responsible for specificity of *L. minor* and *L. punctata* callus culture toxicity assessments. Attempts to utilize *L. minor* callus medium for *L. punctata* callus growth (and visa versa) were unsuccessful, thus preventing analysis of species specificity of callus culture toxicity assessments independent of callus growth medium. Therefore, two plausible explanations for specificity of *L. minor* and *L. punctata* 815 callus culture toxicity assessments were species specificity and callus culture method specificity.

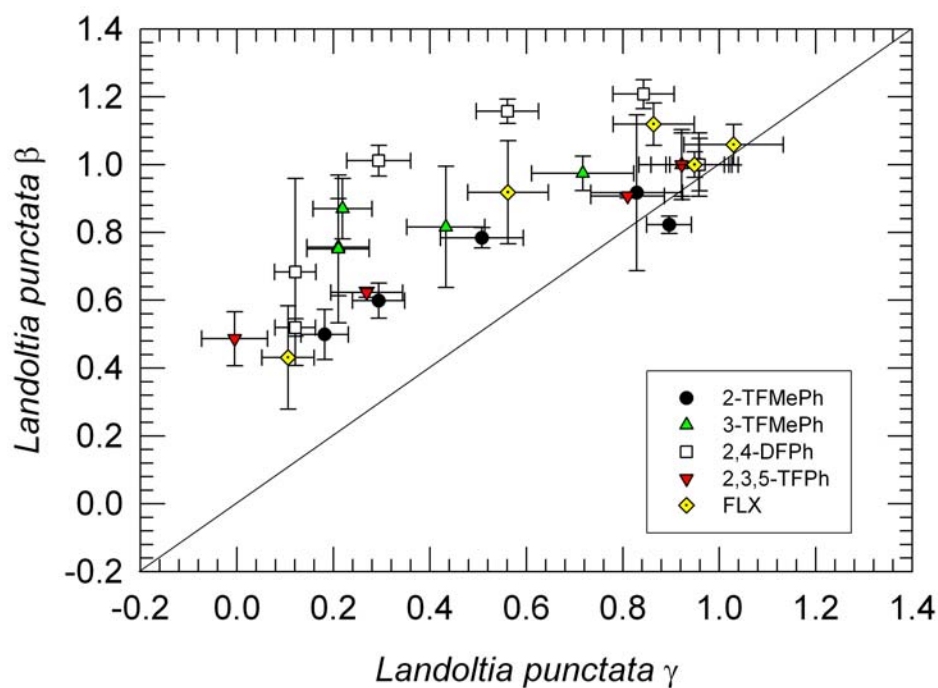
Comparisons of callus culture and OP responses

Trends for fluorinated organic pollutant toxicities in callus and whole plant cultures indicated potential qualitative and comparative applications of callus toxicity assessments, in addition to suggesting that species specificity was responsible for specificity of *L. minor* and *L. punctata* 815 callus culture toxicity assessments. Callus culture toxicity assessments did maintain species specificity observed in OP assessments for 2-TFMeP, 3-TFMeP, 2,4-DFP, and 2,35-TFP. *L. punctata* 815 callus cultures and fronds were more susceptible to inhibition by 3-TFMeP and 2,4-DFP than were *L. minor*

callus cultures and fronds, as indicated by lower LOEC values and/or lower EC50 values. EC50 and LOEC values for 2,3,5-TFP were statistically similar for *L. minor* and *L. punctata* whether determined from callus culture toxicity or OP assessments. Additionally, while statistically similar EC50 values were obtained for *L. minor* and *L. punctata* 815 callus cultures, mean EC50 and LOEC values indicated slightly higher toxicity of 2-TFMeP to *L. minor* callus cultures than *L. punctata* 815 callus cultures; likewise, EC50 and LOEC values for 2-TFMeP exposure to *L. minor* fronds were less than corresponding values for *L. punctata* fronds. The one exception to species-specificity for toxicity of fluorinated organic pollutants was fluoxetine. Fluoxetine, while more toxic to *L. punctata* callus cultures than *L. minor*, was more toxic to *L. minor* fronds than to *L. punctata* fronds, as indicated by lower EC50 and LOEC values. *L. minor* callus growth was stimulated by 5 μM to 100 μM fluoxetine; mean final surface areas for *L. minor* callus cultures exposed to fluoxetine were 7.41 to 7.94 mm^2 , as compared to 6.50 mm^2 for control *L. minor* callus cultures. Consequently, one plausible explanation for non-species specific response to fluoxetine for *L. minor* callus cultures is that fluoxetine possesses cytokinin- or auxin-like activity that enhances culture of *L. minor* callus. Therefore, while fluorinated and trifluoromethylated phenol results indicate that callus culture toxicity assessments can generally be utilized for **qualitative** species-specific comparisons of toxicity in whole plants, fluoxetine results suggest that care should be taken in assuming callus culture toxicities are applicable to whole plant systems when stimulation of callus growth is observed.

Comparison of γ -values for callus culture toxicity assessments with β -values for OP assessments indicated that callus culture toxicity assessments did not quantitatively reflect whole plant toxicity of *L. minor* and *L. punctata* to fluorinated organic pollutants. As shown in Figure 8.5, normalized responses in OP assessments (i.e., β values) were not linearly correlated to normalized responses in callus culture toxicity assessments (i.e., γ

a.)



b.)

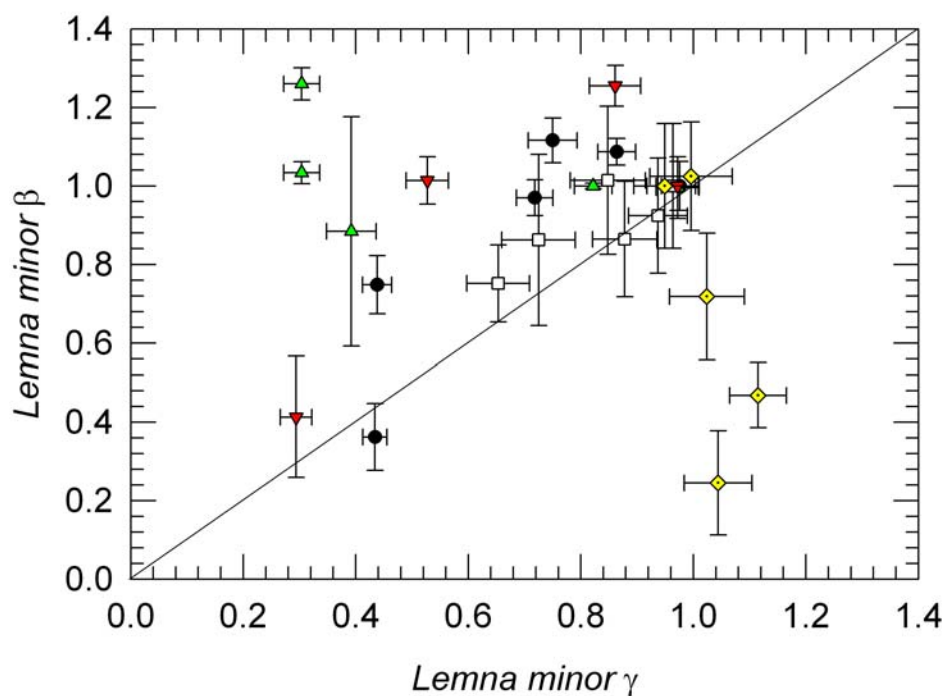


Figure 8.5. Comparison of normalized responses of fronds (β) to normalized responses of callus cultures (γ) to fluorinated organic pollutants for *L. punctata* (a) and *L. minor* (b). Solid line represents $\beta = \gamma$. Points represent mean values and error bars represent standard error of 3 and 48 replicates, respectively.

values) for either *L. punctata* or *L. minor* (i.e., $r^2 = 0.40$ and 0.03 , respectively). Absence of relationships between β and γ , for both *L. punctata* and *L. minor*, strongly suggests that toxicities, as determined in plant tissue cultures, can not be **directly** equated with whole plant toxicity. In general, callus cultures were more susceptible to inhibitory effects of fluorinated organic pollutants than were plants; greater than 76% (*L. minor*) and >96.3% (*L. punctata*) of data points in Figure 8.5 were above the $\beta = \gamma$ line. Uninhibited, and even stimulated, responses of *L. minor* callus cultures to fluoxetine were the major exceptions to increased toxicity of fluorinated organic pollutants in callus culture toxicity assessments (Figure 8.5). These trends were also reflected in EC50 and LOEC values obtained from callus culture toxicity and OP assessments. For all combinations of species and experimental fluorinated and trifluoromethylated phenols, EC50 values for OP assessment were statistically significantly (and substantially) greater than EC50 values for callus culture toxicity assessment, indicating that callus culture toxicity assessments may substantially over predict whole plant toxicity (Table 8.1). Increased susceptibility of callus cultures to fluorinated organic pollutant toxicity is likely a major contributor to lack of relationship between β and γ . As both whole-plant and callus culture toxicities demonstrated sigmoid responses increasing concentrations of fluorinated organic pollutants, the rate of decrease expected for low levels of inhibition is expected to be less than the rate of decrease at moderate levels of inhibition. In other words, β values presented in Figure 8.5 typically represent the OP-concentration trend at the beginning of the sigmoid curve, while γ values represent the log A_{∞} -concentration trends over the entirety of the curve. Potential explanations for increased susceptibility of callus cultures to fluorinated organic pollutant toxicity include absence of plant-level or organ-level regulation of toxicity, absence of epidermal cells, and decreased metabolism of organic pollutants in callus cultures as compared to whole plant systems.

One potential weakness of callus culture toxicity assessments is limited exposure of callus cultures to pollutant because of limited contact between callus cultures and

solidified medium and diffusion gradients within the medium. However, results presented herein strongly indicate that exposure of callus cultures to pollutants was not limited. Based on increased volatility of fluorinated and trifluoromethylated phenols as compared to fluoxetine, one would expect higher exposure of pollutants to fluorinated and trifluoromethylated phenols, resulting in higher relative toxicity (i.e., greater ratios of $\gamma:\beta$). However, the greatest observed $\gamma:\beta$ ratios were for fluoxetine (e.g., ~ 0.6 for *L. punctata* 815 and >2.8 for *L. minor*). In contrast, $\gamma:\beta$ ratios for fluorinated and trifluoromethylated phenols were less than 0.4. Consequently, exposure of callus cultures to fluorinated organic pollutants was not limiting factor in the above toxicity assessments.

Effects of pH on toxicity of fluoxetine to Lemnaceae plants

Based on results from previous studies that indicated inhibition of *L. punctata* OP at concentrations ≤ 10 μM fluoxetine at pH 7 (Chapter 6), effects of fluoxetine exposure on *L. punctata* OP at pH 5.8 (pH value used for OP assessments in this study) and pH 7.0 were assessed (Figure 8.6). Inhibition of *L. punctata* OP, as indicated by β , was observed as concentrations as low as 2 μM at pH 7.0 (i.e., $p < 0.05$, $n = 9$), but was not observed until concentrations reached 50 μM fluoxetine at pH 5.8 (i.e., $p < 0.05$, $n = 9$). Control *L. punctata* OP was similar at pH 5.8 and pH 7.0 (i.e., 12.9 ± 0.5 $\mu\text{mol O}_2/\text{h-g}$ and 13.4 ± 0.4 $\mu\text{mol O}_2/\text{h-g}$, respectively), suggesting that basal plant activity did not contribute to differences observed in fluoxetine toxicity at pH 5.8 and pH 7.0. Additionally, dependence of toxicity of fluoxetine to *L. punctata* was not likely directly attributed to pH. Dissociation constant (pK_a) of fluoxetine is 8.7 (Eli Lilly 2003), indicating that fraction protonated at pH 5.8 versus 7.0 would not substantially differ (i.e., 0.999 vs. 0.980, respectively). However, hydrophobicity of fluoxetine depends on pH, as indicated by log of octanol- water partitioning coefficients ($\log K_{ow}$) of 1.0 at pH 5.0, 1.8 at pH 7.0, and 2.6 at pH 9.0 (Eli Lilly 2003). Therefore, the most probable explanation for dependence of fluoxetine toxicity to *L. punctata* on pH is increased partitioning into *L.*

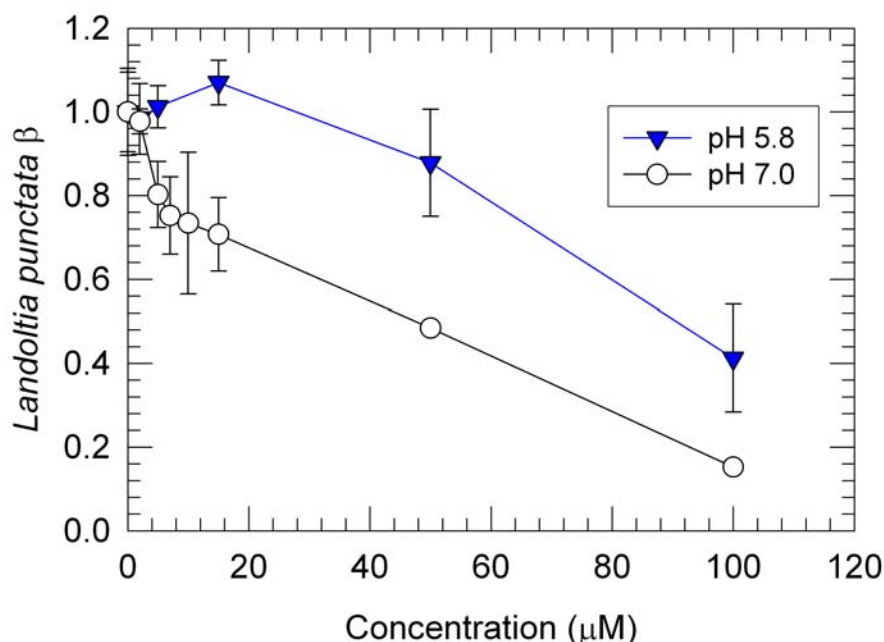


Figure 8.6. Normalized responses of *L. punctata* oxygen production rate (β) as affected by concentration of fluoxetine (μM) at pH 5.8 and pH 7.0. Points represent means and error bars represent standard error of triplicate reactors.

punctata, as indicated by greater $\log K_{OW}$, at higher pH values. Increased partitioning would consequently result in higher internal concentrations of fluoxetine at pH 7.0 than at pH 5.8 despite similar external concentrations.

Phytometabolism of 3-TFMePh in Lemnaceae plants and callus

Phytometabolism of 3-TFMeP by Lemnaceae plants and callus cultures yielded similar metabolites, as shown in Figure 8.7. Parent 3-TFMeP eluted at a retention time of ~ 34.3 min and was observed in all samples. A substantial peak at 28.5 min was observed in extracts of *L. punctata* and *L. minor* callus cultures and was identified as 2,4-D, a phytohormone that was a component of both callus culture media, by comparison when standard reference retention, UV-Vis spectra, and mass spectra. When total ion chromatograms were extracted for 161 mass to charge ratio (m/z), one peak at 13.2 min was observed in all samples. UV/Vis and mass spectra of peak at 13.2 min were similar

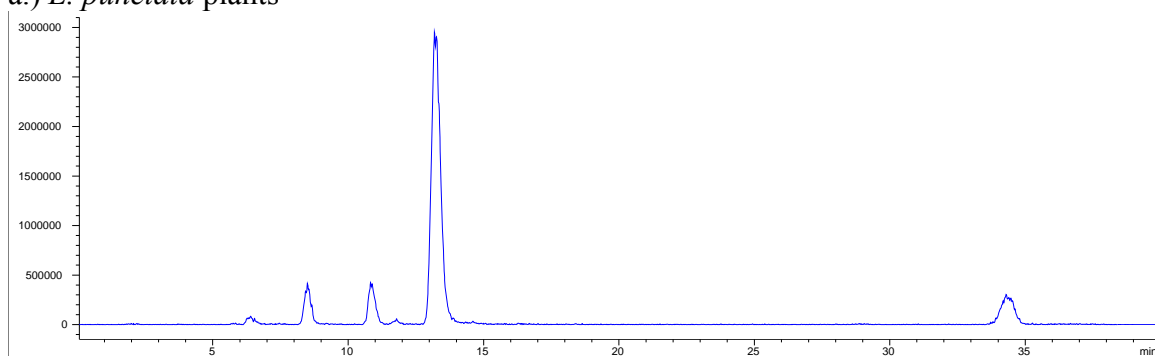
for both Lemnaceae plants and callus cultures and variations in retention times were negligible (i.e., <0.1 min). An example mass spectrum of the peak at 13.2 is provided in Figure 8.8. Fractionation of 3-TFMeP metabolite from electrospray ionization (negative mode) yielded m/z of 161, 365, 409, and 819, which was consistent with a malonyl-glucopyranoside conjugate of 3-TFMeP. Malonyl-glucopyranoside conjugates have been previously associated with phytometabolism of chlorinated phenols by *L. minor* and demonstrated similar fractionation m/z under negative electrospray ionization (Day and Saunders 2004). Additionally, malonyl-glucopyranoside conjugates of halogenated phenols typically result from conjugation with glucopyranoside followed by subsequent conjugation with malonyl residue, as hypothesized in Figure 8.8 for 3-TFMeP.

Conjugation of 3-TFMeP with glucopyranoside was observed at levels near detection limits for whole plant cultures and was not observed in callus cultures, indicating that this intermediate metabolite may be transient in both whole plant and callus cultures.

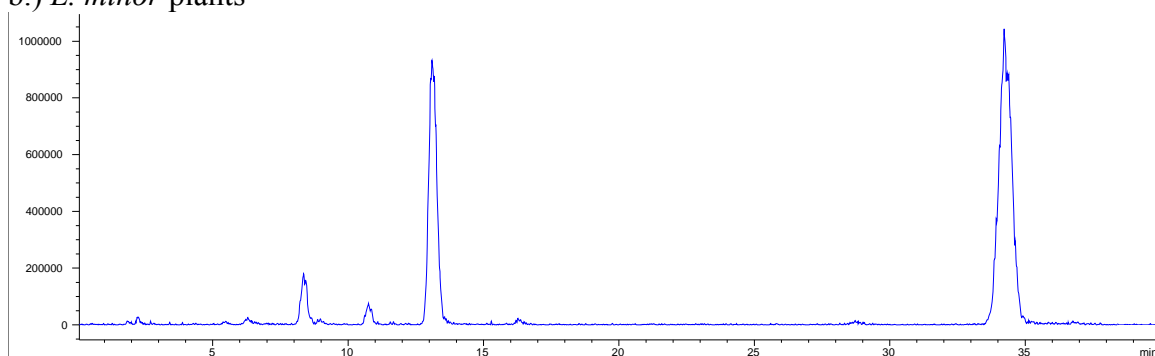
Furthermore, metabolism of 3-TFMeP via conjugation with malonyl-glucopyranoside strongly suggests that 3-TFMeP is sequestered into plant vacuoles, as malonyl residues signal vacuolar compartmentalization in plants (Komossa et al. 1995; Day and Saunders 2004). Products of other potential plant metabolism pathways, including glutathione conjugation and transformation, were not observed in plant or callus culture extracts.

Fractionation of 3-TFMeP metabolite primarily yielded m/z of 161, suggesting that 3-trifluoromethylphenyl moiety was released upon electrospray ionization and facilitating comparisons between metabolite and parent 3-TFMeP peaks. With the exception of *L. minor* plants, 3-TFMeP metabolite peak areas were greater than parent 3-TFMeP peak areas, indicating that concentration of 3-TFMeP metabolites exceeded internal concentration of 3-TFMeP. However, metabolism of 3-TFMeP in *L. minor* plants was limited, as indicated by a relatively small peak for 3-TFMeP metabolite. Observations of *L. minor* health at conclusion of 5-d exposure for metabolite identification indicated that *L. minor* was in poor health; approximately 50% of *L. minor* fronds were brown and

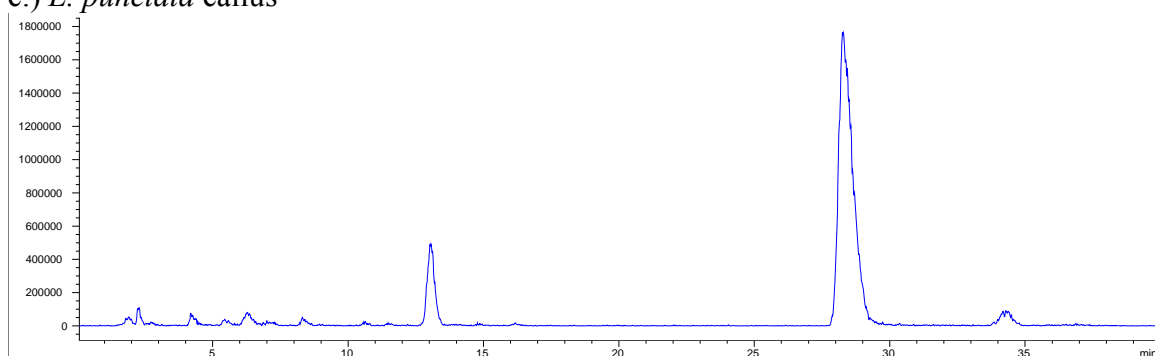
a.) *L. punctata* plants



b.) *L. minor* plants



c.) *L. punctata* callus



d.) *L. minor* callus

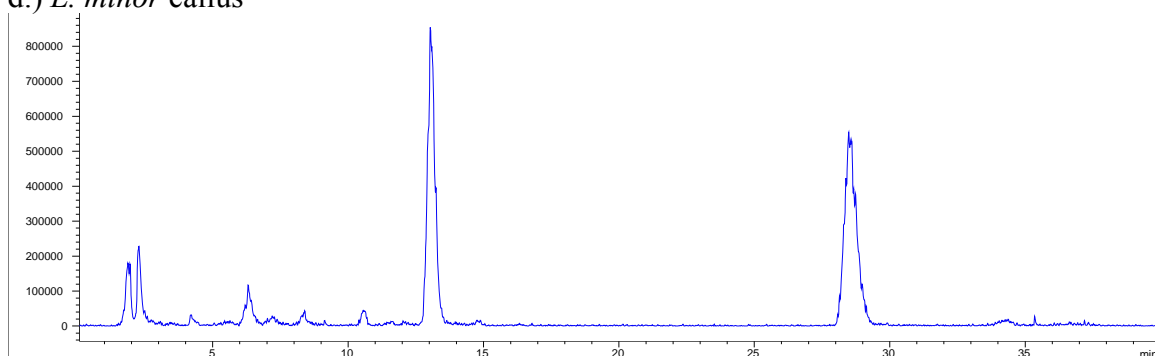


Figure 8.7. Example chromatographic traces of (a.) *L. punctata* plants, (b.) *L. minor* plants, (c.) *L. punctata* callus cultures, and (d.) *L. minor* callus cultures exposed to 3-TFMeP. Scans represent extraction of 161 m/z from total ion.

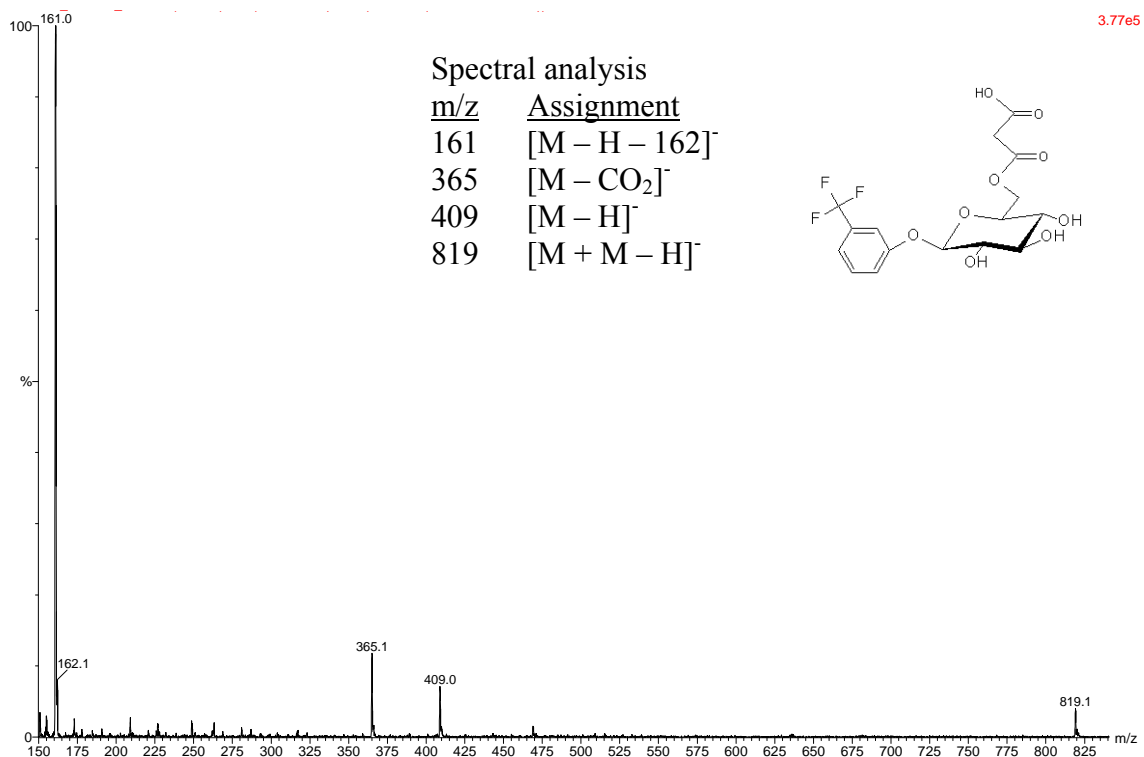


Figure 8.8. Mass spectra of 3-TFMeP metabolite peak (retention time =22.6 min) in *L. punctata* plant extraction. Spectral analysis, including observed mass to charge ratios (m/z) and assignments, and structure of proposed malonyl-gluocopyranoside metabolite of 3-TFMeP consistent with spectral analysis is provided.

mottled. Poor health was attributed to external factors beyond 3-TFMeP exposure, as *L. minor* that was not exposed to 3-TFMeP also exhibited poor health. Additionally, uptake of 3-TFMeP was limited in *L. minor* exposures (i.e., 39.2% vs. 64.3% for *L. punctata* exposures). In contrast to *L. minor* plants, *L. punctata* plants, *L. punctata* callus cultures, and *L. minor* callus cultures accumulated 3-TFMeP metabolites in concentrations greater than internal concentration of 3-TFMeP. Peak heights for 3-TFMeP metabolites in *L. punctata* plants, *L. punctata* callus cultures, and *L. minor* callus cultures were approximately 2.9, 1.8 and 1.3 greater than peak heights for 3-TFMeP (data not shown), indicating accumulation of 3-TFMeP metabolites in both whole plant and callus cultures.

Summary

Research presented herein advances use of callus cultures for assessment of phytotoxicities and phytometabolism of organic pollutants by aquatic plants. Callus culture toxicity responses were species-specific for fluorinated and trifluoromethylated phenols, but not for fluoxetine, suggesting that callus culture toxicity assessments are qualitatively and comparatively applicable to whole plant toxicity when stimulatory effects from pollutant are not observed. However, callus cultures exhibited greater susceptibility to fluorinated organic pollutant toxicity than whole plant cultures, as measured by EC₅₀, LOEC, and γ or β values. Consequently, responses of callus cultures and whole plant cultures at given concentrations of fluorinated organic pollutants were not correlated, indicating that direct, quantitative application of callus culture toxicity assessments to whole plant toxicity is not warranted.

Phytometabolism of 3-TFMeP in Lemnaceae plant and callus cultures was consistent with conjugation with malonyl-glucopyranoside and subsequent sequestration of 3-TFMeP metabolite into vacuoles. Similar metabolic profiles were observed for *L. minor* and *L. punctata* fronds, indicating that metabolism of 3-TFMeP was similar in both species of duckweed. Additionally, similar metabolic profiles were observed for callus cultures and whole plants. Results support further research into development of callus cultures to study metabolism of organic pollutants in aquatic plant systems.

CHAPTER 9: CONCLUSIONS AND RECOMMENDATIONS

In natural and engineered systems, aquatic plants actively uptake organic pollutants. Characterization of organic pollutant uptake by aquatic plants is essential for increased understanding of pollutant fate and improved design of plant-based treatment systems for polluted surface waters. Research presented in this dissertation examined uptake of fluorinated phenols, effects of concentration and temperature on uptake, contributions of uptake and passive plant-associated processes to aqueous depletion of wastewater-associated organic pollutants, and application of callus cultures to toxicity and metabolism of fluorinated organic pollutants in Lemnaceae plants.

Uptake of fluorinated phenols by Lemnaceae was rapid, with pseudo-first-order uptake rates of $0.20 \pm 0.04 - 0.84 \pm 0.07 \text{ d}^{-1}$. Uptake rates of fluorinated phenols were pollutant-specific and appeared to depend on factors affecting rates of metabolism of organic pollutants. Attempts to correlate uptake rates with pollutant properties, including K_{OW} , pK_a , and Hammett's constants, indicated that uptake rates were independent of commonly used pollutant properties. Positioning and type of fluoro-substituents on the phenol ring were the most indicative parameters for uptake rates. *L. minor* uptake rates for monotrifluoromethylphenols were generally greater than uptake rates for monofluorophenols. Additionally, uptake rates for *ortho*, *ortho* substituted fluorophenols were less than uptake rates for fluorophenols with the same number of fluoro-substituents. Although uptake rates were not correlated with known pollutant properties, uptake rates indicated that plant metabolism of organic pollutants plays an important role in sustaining uptake of fluorinated phenols by Lemnaceae.

To determine effects of concentration on uptake of fluorinated phenols by *L. minor*, an oxygen production rate (OP) assessment was developed to quantify inhibitory effects of fluorinated phenols on *L. minor*. OP assessment was developed with consideration of experimental parameters that may inhibit OP by *L. minor*, including concentrations of carbonate and phosphate buffer in the media, time of assessment, and mass of *L. minor*. The OP by *L. minor* was inhibited by increasing media pH and mass of *L. minor*. The developed OP assessment utilized 0.5 g *L. minor* per 60 ml of modified Standard Methods medium. A total of 30 h was needed to determine toxicity of fluorinated phenols, with an exposure period of 24 h, an incubation time of 6 h, and negligible analysis time. At concentrations of 10 to 1750 μM , 3-trifluoromethylphenol (3-TFMeP) exhibited a sigmoid toxicity response with an EC_{50} of $675 \pm 37 \mu\text{M}$ 3-TFMeP. However, 3-fluorophenol (3-FP) was neither toxic nor inhibitory at concentrations less than 1750 μM . Substantial decreases in uptake rate constants were observed for increasing concentration of both fluorinated phenols, indicating that concentration may be a more important indicator of uptake by *L. minor* than decreasing plant activity due to toxicity.

Uptake rates of fluorinated phenols also depended on temperature, with increases in uptake rates with increasing temperatures following pollutant-specific Arrhenius relationships. Mass of fluorinated phenol sorbed to *L. minor* plants was independent of temperature, indicating that increased partitioning of fluorinated phenols into or onto *L. minor* did not contribute to increased aqueous depletion of fluorinated phenols in Lemnaceae systems. Plant activity generally increased with temperatures of 12°C to 27°C; however, trends of increasing plant activity and increasing plant uptake of

fluorinated phenols with temperature were not similar, suggesting that increases in uptake rates with temperature were not wholly attributable to plant activity. Inhibition of *L. minor* at 500 μ M fluorinated phenol also depended on temperature, with increased inhibitory effects observed at 12°C and 35°C. Nonetheless, results indicated that temperature directly affected uptake of fluorinated phenols, likely through effects on enzymatic processing, as contributions of sorption, plant activity, and plant inhibition to temperature effects on uptake were minimal.

Uptake is a process that incorporates passive abiotic partitioning into plants and active plant metabolism of internal organic pollutants. Metabolism of 3-TFMeP proceeded through glucosylation and subsequent malonyl conjugation; similar metabolic pathways have been observed for chlorophenols (Day and Saunders 2004) and are reasonably assumed for fluorinated phenols. Steric hindrance of enzymes responsible for glucosylation would explain observed adverse effects of *ortho*, *ortho*- substitution of phenol on uptake rates. Independence of uptake rates on hydrophobicity (i.e., K_{OW}) emphasized role of plant metabolism in uptake of fluorinated phenols, while independence of uptake rate on molecular properties (i.e., Hammett's constants) suggests that multiple isoenzymes may be responsible for metabolism of fluorinated phenols in Lemnaceae. Additionally, trends for increasing uptake rates with increasing temperature, as represented by pollutant-specific Arrhenius relationships, further confirmed importance of plant metabolism in uptake (as sorption was constant with temperature). Therefore, research on uptake of fluorinated phenols supported the hypothesis that metabolism rates limited, and thus, determined uptake rates of fluorinated organic pollutants.

While care must be taken in extrapolating laboratory results presented in this dissertation to full-scale wetlands, research imparted insights into effects of environmental conditions on uptake of fluorinated organic pollutants by aquatic plants in wetlands. Decrease in uptake rates with increasing concentrations of 10 μM to 1750 μM fluorinated phenol suggest that uptake rates presented in this dissertation for fluorinated phenols (i.e., $0.020 \pm 0.04 - 0.84 \pm 0.07 \text{ d}^{-1}$) underestimate uptake rates in wetland systems with significantly lower concentrations of fluorinated organic pollutants (i.e., typically nM concentrations). Additionally, while effects of temperature on uptake rates were pollutant-specific, results demonstrated that (1) uptake rates increased with increasing temperature and that (2) uptake of organic pollutants by Lemnaceae occurs over a broad range of temperatures (i.e., 12°C to 35°C). Direct effects of concentration and temperature more greatly influenced uptake rates than did indirect effects of concentration and temperature on inhibition and plant activity (respectively); consequently, concentration and temperature effects were considered an intrinsic characteristic of uptake of fluorinated organic pollutants by Lemnaceae and thus important environmental conditions that will affect fate and removal of fluorinated organic pollutants in wetlands.

Examining uptake of fluorinated phenols (e.g., a suite of similarly structured small chemicals) facilitated studies on effects of pollutant properties, pollutant concentration, and water temperature; however, examination of uptake of more complex chemicals that are representative of organic pollutants in wastewaters was required to contextualize role of uptake by Lemnaceae in fate of organic pollutants in wetlands. Of six wastewater-associated organic pollutants, only fluoxetine was uptaken by Lemnaceae.

To a lesser extent, active plant uptake also indirectly affected aqueous depletion of triclosan and 2,4-dichlorophenoxyacetic acid via uptake of 2,4-dichlorophenol, a degradation product of triclosan and 2,4-D. Presence of Lemnaceae enhanced microbial degradation of ibuprofen, most likely through provision of organic carbon that increased microbial activity or supported cometabolic microbial degradation. Sorption to Lemnaceae tissues was an important process in aqueous depletion of fluoxetine and triclosan in Lemnaceae systems. In sum, active and passive plant-associated processes affected fate of four of six wastewater-associated organic pollutants, with active plant uptake directly and indirectly affecting fate of three of six organic pollutants. Role of uptake was substantially reduced for wastewater-associated organic pollutants than for fluorinated phenols (i.e., 3 of 6 vs. 13 of 14 actively uptaken by Lemnaceae); however, results indicated that active plant uptake by Lemnaceae and passive plant-associated processes significantly contribute to fate and removal of many wastewater-associated organic pollutants in wetlands.

Toxicity and metabolism studies with Lemnaceae callus cultures indicated qualitative applicability of callus cultures to whole plant toxicity and metabolism. Callus cultures were more susceptible to toxicity from four fluorinated phenols than were Lemnaceae plants, but were less susceptible to toxicity from fluoxetine than were Lemnaceae plants. With the exception of fluoxetine, *L. minor* and *L. punctata* callus cultures maintained species-specificity with regards to toxicity. Decreased susceptibility to inhibition by fluoxetine in callus cultures was attributed to stimulation of callus growth by fluoxetine. Consequently, callus culture toxicity assessments were considered qualitatively and comparatively applicable to whole plant toxicity. Analysis of extracts

of *L. minor* plants, *L. punctata* plants, *L. minor* callus cultures, and *L. punctata* callus cultures exposed to 3-trifluoromethylphenol yielded similar chromatograms, showing parent 3-trifluoromethylphenol and one metabolite. Based on mass spectral analysis of extracts and previous research on metabolism of chlorophenols by *L. minor* (Day and Saunders 2004), Lemnaceae plants and callus metabolized 3-trifluoromethylphenol through conjugation of 3-trifluoromethylphenol with glucopyranoside and malonyl residues. Therefore, comparison between metabolism of 3-trifluoromethylphenol in Lemnaceae plants and Lemnaceae callus cultures indicated that callus culture results are applicable to whole plant metabolism of organic pollutants.

Comparisons between toxicities and metabolism of fluorinated organic pollutants in Lemnaceae callus cultures and Lemnaceae plants were completed to evaluate applicability of callus culture results to whole plants in multiple species (i.e., *L. punctata* and *L. minor*) collected from constructed treatment wetlands. Simplicity of toxicity, uptake, and metabolism assessments with Lemnaceae plants due to diminutive physiology diminishes the need for Lemnaceae callus culture assessments. For example, callus culture toxicity assessments required substantially greater replicates (i.e., 48) than did oxygen production rate assessments (i.e., 3) because of large variability in callus culture growth. Assessment of fluorinated organic pollutant toxicity in callus cultures required a longer exposure time (i.e., 28-d) than did assessment of toxicity in Lemnaceae plants (i.e., 2-d). Additionally, extractions of whole Lemnaceae populations are realistic and simple, particularly when compared to extractions of larger plants like cattails, reeds, and cottonwood trees. Finally, successful induction and growth of callus in Lemnaceae species from constructed treatment wetlands were achieved for only two of six published

procedures. However, while callus culture assessments may not be the preferred method for determining toxicity and metabolism in Lemnaceae species, results support use of callus culture assessments in plant species with more complex physiology and larger size than Lemnaceae species.

Recommendations for future research

Research presented within this dissertation indicated that plant uptake of fluorinated phenols and of many wastewater-associated organic pollutants by Lemnaceae plays an important role in fate and removal of organic pollutants. Studies on effects of concentration and temperature on uptake advanced scientific understanding of effects of uncontrollable environmental parameters on uptake rates of fluorinated organic pollutants. Furthermore, multiple contributions of plants to fate and removal of wastewater-associated organic pollutants, through plant uptake, plant enhancement of microbial degradation, and sorption to plant tissues, were demonstrated, epitomizing complex nature of interactions of organic pollutants and aquatic plants. Research also documented qualitative applicability of toxicity and metabolism results obtained from Lemnaceae callus cultures to Lemnaceae plants.

Additional research is warranted to more fully understand role of aquatic plants in fate and removal of organic pollutants in wetlands. Development of wetland mesocosms that closely represent conditions in natural and constructed treatment wetlands (e.g., multiple plant species, heterogeneous redox conditions in rhizosphere, presence of organic matter in waters, representative mixtures of organic pollutants) would be beneficial in understand fate and future development of wetlands for treatment and reclamation of wastewaters. Characterization of the *Arabidopsis thaliana* genome (The

Arabidopsis Genome Initiative 2000) and isolation of plant glucosyltransferases (Loutre et al. 2003) in recent years have greatly advanced understanding of plant metabolism of organic pollutants. As new genomic and proteomic tools are developed in the future, the role of enzymatic processing in determination of uptake rates for fluorinated phenols should be reinvestigated and directly confirmed. Studies that examined roles of active and passive plant processes in fate of wastewater-associated organic pollutants should be expanded to include more pharmaceuticals, personal care products, and pesticides. Additionally, plant metabolism of wastewater-associated organic pollutants that are uptaken by Lemnaceae plants should be characterized, particularly for fluoxetine. Metabolism of organic pollutants in plant callus and cell suspension cultures should be evaluated for additional species of wetland plants, including emergent, rooted, and submerged aquatic plants.

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